BH

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 9/22, 15/55, 15/70

A1

(11) International Publication Number: WO 95/09233

(43) International Publication Date: 6 April 1995 (06.04.95)

(21) International Application Number: PCT/US94/09143

(22) International Filing Date: 23 August 1994 (23.08.94)

(30) Priority Data:
08/126,564 27 September 1993 (27.09.93) US

(81) Designated States: AU, CA, JP, NZ, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(71) Applicant: THE JOHNS-HOPKINS UNIVERSITY [US/US]; 34th & Charles Streets, Baltimore, MD 21218 (US).

(72) Inventor: CHANDRASEGARAN, Srinivasan; 4 East 32nd Street #206, Baltimore, MD 21218 (US).

(74) Agents: KOKULIS, Paul, N. et al.; Cushman, Darby & Cushman, 1100 New York Avenue, N.W., Washington, DC 20005 (US).

(54) Title: FUNCTIONAL DOMAINS IN *FLAVOBACTERIUM OKEANOKOITES* (FOKI) RESTRICTION ENDONUCLEASE

(57) Abstract

The present inventors have identified the recognition and cleavage domains of the *FokI* restriction endonuclease. Accordingly, the present invention relates to DNA segments encoding the recognition and cleavage domains of the *FokI* restriction endonuclease, respectively. The 41 kDa N-terminal fragment constitutes the *FokI* recognition domain while the 25 kDa C-terminal fragment constitutes the *FokI* cleavage nuclease domain. The present invention also relates to hybrid restriction enzymes comprising the nuclease domain of the *FokI* restriction endonuclease linked to a recognition domain of another enzyme. One such hybrid restriction enzyme is *Ubx-F*$_N$. This enzyme contains the homeo domain of *Ubx* linked to the cleavage or nuclease domain of *FokI*. Additionally, the present invention relates to the construction of two insertion mutants of *FokI* endonuclease.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgystan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SI	Slovenia
CI	Côte d'Ivoire	KZ	Kazakhstan	SK	Slovakia
CM	Cameroon	LI	Liechtenstein	SN	Senegal
CN	China	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	LV	Latvia	TJ	Tajikistan
DE	Germany	MC	Monaco	TT	Trinidad and Tobago
DK	Denmark	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali	UZ	Uzbekistan
FR	France	MN	Mongolia	VN	Viet Nam
GA	Gabon				

FUNCTIONAL DOMAINS IN *FLAVOBACTERIUM OKEANOKOITES* (FOKI) RESTRICTION ENDONUCLEASE

BACKGROUND OF THE INVENTION

1. Field of the Invention:

The present invention relates to the *Fok*I restriction endonuclease system. In particular, the present invention relates to DNA segments encoding the separate functional domains of this restriction endonuclease system.

The present invention also relates to the construction of two insertion mutants of *Fok*I endonuclease.

Additionally, the present invention relates to a hybrid enzyme (Ubx-F_N) prepared by linking the *Ultrabithorax Ubx* homeo domain to the cleavage domain (F_N) of *Fok*I.

2. Background Information:

Type II endonucleases and modification methylases are bacterial enzymes that recognize specific sequences in duplex DNA. The endonuclease cleaves the DNA while the methylases methylate adenine or cytosine residues so as to protect the host-genome against cleavage [Type II restriction

and modification enzymes. In Nucleases (Eds. Modrich and Roberts) Cold Spring Harbor Laboratory, New York, pp. 109-154, 1982]. These restriction-modification (R-M) systems function to protect cells from infection by phage and plasmid molecules that would otherwise destroy them.

As many as 2500 restriction enzymes with over 200 specificities have been detected and purified (Wilson and Murray, Annu. Rev. Genet. 25:585-627, 1991). The recognition sites of most of these enzymes are 4-6 base pairs long. The small size of the recognition sites is beneficial as the phage genomes are usually small and these small recognition sites occur more frequently in the phage.

Eighty different R-M systems belonging to the Type IIS class with over 35 specificities have been identified. This class is unique in that the cleavage site of the enzyme is separate from the recognition sequence. Usually the distance between the recognition site and the cleavage site is quite precise (Szybalski et al., Gene, 100:13-26, 1991). Among all these enzymes, the *Fok*I restriction endonuclease is the most well characterized member of the Type IIS class. The *Fok*I endonuclease (*RFokI*) recognizes asymmetric pentanucleotides in double-stranded DNA, 5′ GGATG-3′ (SEQ ID NO: 1) in one strand and 3′-CCTAC-5′ (SEQ ID NO: 2) in the other, and introduces staggered cleavages at sites away from the recognition site (Sugisaki et al., Gene 16:73-78; 1981). In contrast, the *Fok*I methylase (*MFokI*) modifies DNA thereby rendering the DNA resistant to digestion by *Fok*I endonuclease. The *Fok*I restriction and modification genes have been cloned and their nucleotide sequences deduced (Kita et al., J. of Biol. Chem., 264:575-5756, 1989). Nevertheless, the domain structure of the

*Fok*I restriction endonuclease remains unknown, although a three domain structure has been suggested (Wilson and Murray, Annu. Rev. Genet. 25:585-627, 1991).

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide isolated domains of Type IIS restriction endonuclease.

It is another object of the present invention to provide hybrid restriction enzymes which are useful for mapping and sequencing of genomes.

An additional object of the present invention is to provide two insertion mutants of *FOKI* which have an increased distance of cleavage from the recognition site as compared to the wild-type enzyme. The polymerase chain reaction (PCR) is utilized to construct the two mutants.

Various other objects and advantages of the present invention will become obvious from the drawings and the following description of the invention.

In one embodiment, the present invention relates to a DNA segment encoding the recognition domain of a Type IIS endonuclease which contains the sequence-specific recognition activity of the Type IIS endonuclease or a DNA segment encoding the catalytic domain of a Type IIS endonuclease which contains the cleavage activity of the Type IIS endonuclease.

In another embodiment, the present invention relates to an isolated protein consisting essentially of the N-terminus or recognition domain of the *Fok*I restriction endonuclease which protein has the sequence-specific recognition activity of

the endonuclease or an isolated protein consisting essentially of the C-terminus or catalytic domain of the *Fok*I restriction endonuclease which protein has the nuclease activity of the endonuclease.

In a further embodiment, the present invention relates to a DNA construct comprising a first DNA segment encoding the catalytic domain of a Type IIS endonuclease which contains the cleavage activity of the Type IIS endonuclease; a second DNA segment encoding a sequence-specific recognition domain other than the recognition domain of the Type IIS endonuclease; and a vector. In the construct, the first DNA segment and the second DNA segment are operably linked to the vector to result in the production of a hybrid restriction enzyme. The linkage occurs through a covalent bond.

Another embodiment of the present invention relates to a procaryotic cell comprising a first DNA segment encoding the catalytic domain of a Type IIS endonuclease which contains the cleavage activity of said Type IIS endonuclease; a second DNA segment encoding a sequence-specific recognition domain other than the recognition domain of said Type IIS endonuclease; and a vector. The first DNA segment and the second DNA are operably linked to the vector such that a single protein is produced. The first DNA segment may encode, for example, the catalytic domain (F_N) of *FokI*, and the second segment may encode, for example, the homeo domain of *Ubx*.

In another embodiment, the present invention relates to a hybrid restriction enzyme comprising the catalytic domain of a Type IIS endonuclease which contains the cleavage activity of the Type IIS endonuclease linked to a recognition domain of an enzyme or a protein other than the Type IIS endonuclease from which the cleavage domain is obtained.

In a further embodiment, the present invention relates to a DNA construct comprising a first DNA segment encoding the catalytic domain of a Type IIS endonuclease which contains the cleavage activity of the Type IIS endonuclease; a second DNA segment encoding a sequence-specific recognition domain other than the recognition domain of the Type IIS endonuclease; a third DNA segment comprising one or more codons, wherein the third DNA segment is inserted between the first DNA segment and the second DNA segment; and a vector. Preferably, the third segment contains four or seven codons.

In another embodiment, the present invention relates to a procaryotic cell comprising a first DNA segment encoding the catalytic domain of a Type IIS endonuclease which contains the cleavage activity of the Type IIS endonuclease; a second DNA segment encoding a sequence-specific recognition domain other than the recognition domain of the Type IIS endonuclease; a third DNA segment comprising one or more codons, wherein the third DNA segment is inserted between the first DNA segment and the second DNA segment; and a vector. The first DNA segment and the second DNA segment are operably linked to the vector so that a single protein is produced.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows sequences of the 5′ and 3′ primers used to introduce new translation signals into *fokIM* and *fokIR* genes during PCR amplification. (SEQ ID NOs: 3-9). SD represents Shine-Dalgarno consensus RBS for *Escherichia coli* (*E. coli*) and 7-bp spacer separates the RBS from the ATG start condon. The *fokIM* primers are flanked by *NcoI* sites. The *fokIR* primers are flanked by *BamHI*

sites. Start and stop codons are shown in bold letters. The 18-bp complement sequence is complementary to the sequence immediately following the stop codon of *MfokI* gene.

FIGURE 2 shows the structure of plasmids pACYC*MfokIM*, pRRS*fokIR* and pCB*fokIR*. The PCR-modified *fokIM* gene was inserted at the *NcoI* site of pACYC184 to form pACYC*fokIM*. The PCR-generated *fokIR* gene was inserted at the *BamHI* sites of pRRS and pCB to form pRRS*fokIR* and pCB*fokIR*, respectively. pRRS possesses a *lac* UV5 promoter and pCB contains a strong *tac* promoter. In addition, these vectors contain the positive retroregulator sequence downstream of the inserted *fokIR* gene.

FIGURE 3 shows SDS (0.1%) - polyacrylamide (12%) gel electrophoretic profiles at each step in the purification of *Fok*I endonuclease. Lanes: 1, protein standards; 2, crude extract from uninduced cells; 3, crude extract from cells induced with 1 mM IPTG; 4, phosphocellulose pool; 5, 50-70% $(NH_4)_2SO_4$ fractionation pool; and 6, DEAE pool.

FIGURE 4 shows SDS (0.1%) - polyacrylamide (12%) gel electrophoretic profiles of tryptic fragments at various time points of trypsin digestion of *Fok*I endonuclease in presence of the oligonucleotide DNA substrate, d-5'-CCTCTGGATGCTCTC-3'(SEQ ID NO: 10): 5'-GAGAGCATCCAGAGG-3'(SEQ ID NO:11). Lanes: 1, protein standards; 2, *Fok*I endonuclease; 3, 2.5 min; 4, 5 min; 5, 10 min; 6, 20 min; 7, 40 min; 8, 80 min; 9, 160 min of trypsin digestion respectively. Lanes 10-13: HPLC purified tryptic fragments. Lanes: 10, 41 kDa fragment; 11, 30 kDa fragment; 12, 11 kDa fragment; and 13, 25 kDa fragment.

FIGURE 5 shows the identification of DNA binding tryptic fragments of *Fok*I endonuclease using

an oligo dT-cellulose column. Lanes: 1, protein standards, 2, *Fok*I endonuclease; 3, 10 min trypsin digestion mixture of *Fok*I - oligo complex; 4, tryptic fragments that bound to the oligo dT-cellulose column; 5, 160 min trypsin digestion mixture of *Fok*I - oligo complex; 6, tryptic fragments that bound to the oligo dT-cellulose column.

FIGURE 6 shows an analysis of the cleavage properties of the tryptic fragments of *Fok*I endonuclease.

(A) The cleavage properties of the tryptic fragments were analyzed by agarose gel electrophoresis. 1 μg of pTZ19R in 10mM Tris.HCl (pH 8), 50mM NaCl, 1mM DTT, and 10mM $MgCl_2$ was digested with 2 μl of the solution containing the fragments (tryptic digests, breakthrough and eluate respectively) at 37°C for 1 hr in a reaction volume of 10 μl. Lanes 4 to 6 correspond to trypsin digestion of *Fok I*- oligo complex in absence of $MgCl_2$. Lanes 7 to 9 correspond to trypsin digestion of *Fok*I - oligo complex in presence of 10 mM $MgCl_2$. Lanes: 1, 1 kb ladder; 2, pTZ19R; 3, pTZ19R digested with *Fok*I endonuclease; 4 and 6, reaction mixture of the tryptic digests of *Fok*I - oligo complex; 5 and 7, 25 kDa C-terminal fragment in the breakthrough volume; 6 and 9, tryptic fragments of *Fok*I that bound to the DEAE column. The intense bands at bottom of the gel correspond to excess oligonucleotides.

(B) SDS (0.1%) - polyacrylamide (12%) gel electrophoretic profiles of fragments from the DEAE column. Lanes 3 to 5 correspond to trypsin digestion of *Fok*I - oligo complex in absence of $MgCl_2$. Lanes 6 to 8 correspond to trypsin digestion of *Fok*I - oligo complex in presence of 10 mM $MgCl_2$. Lanes: 1, protein standards; 2, *Fok*I endonuclease;

3 and 6, reaction mixture of the tryptic digests of *Fok*I - oligo complex; 4 and 7, 25 kDa C-terminal fragment in the breakthrough volume; 5 and 8, tryptic fragments of *Fok*I that bound to the DEAE column.

FIGURE 7 shows an analysis of sequence - specific binding of DNA by 41 kDa N-terminal fragment using gel mobility shift assays. For the exchange reaction, the complex (10 μl) was incubated with 1 μl of ^{32}P-labeled specific (or non-specific) oligonucleotide duplex in a volume of 20 μl containing 10 mM Tris.HCl, 50 mM NaCl and 10 mM $MgCl_2$ at 37°C for various times. 1 μl of the 5'-^{32}P-labeled specific probe [d-5'-CCTCTGGATGCTCTC-3'(SEQ ID NO: 10): 5'-GAGAGCATCCAGAGG-3' (SEQ ID NO: 11)] contained 12 picomoles of the duplex and ~ 50 x 10^3 cpm. 1μl of the 5'-^{32}P-labeled non-specific probe [5'-TAATTGATTCTTAA-3'(SEQ ID NO: 12):5'-ATTAAGAATCAATT-3' (SEQ ID NO: 13)] contained 12 picomoles of the duplex and ~ 25 x 10^3 cpm. (A) Lanes: 1, specific oligonucleotide duplex; 2, 41 kDa N-terminal fragment-oligo complex; 3 and 4, specific probe incubated with the complex for 30 and 120 min respectively. (B) Lanes: 1, non-specific oligonucleotide duplex; 2, 41 kDa N-terminal fragment-oligo complex; 3 and 4 non-specific probe incubated with the complex for 30 and 120 min respectively.

FIGURE 8 shows SDS (0.1%) polyacrylamide (12%) gel electrophoretic profiles of tryptic fragments at various time points of trypsin digestion of *Fok*I endonuclease. The enzyme (200 μg) in a final volume of 200 μl containing 10 mM Tris.HCl, 50 mM NaCl and 10mM $MgCl_2$ was digested with trypsin at RT. The trypsin to *Fok*I ratio was 1:50 by weight. Aliquots (28 μl) from the reaction mixture removed at different time intervals and

quenched with excess antipain. Lanes: 1, protein standards; 2, *Fok*I endonuclease; 3, 2.5 min; 4, 5.0 min; 5, 10 min; 6, 20 min; 7, 40 min; 8, 80 min; and 9,160 min of trypsin digestion respectively.

FIGURE 9 shows the tryptic map of *Fok*I endonuclease (A) *Fok*I endonuclease fragmentation pattern in absence of the oligonucleotide substrate. (B) *Fok*I endonuclease fragmentation pattern in presence of the oligonucleotide substrate.

FIGURE 10 shows the predicted secondary structure of *Fok*I based on its primary sequencing using the PREDICT program (see SEQ ID NO:31). The trypsin cleavage site of *FokI* in the presence of DNA substrates is indicated by the arrow. The KSELEEKKSEL segment is highlighted. The symbols are as follows: h, helix; s, sheet; and •, random coil.

FIGURE 11 shows the sequences of the 5′ and 3′ oligonucleotide primers used to construct the insertion mutants of *Fok*I (see SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38 and SEQ ID NO:39, respectively). The <u>four</u> and <u>seven</u> codon inserts are shown in bold letters. The amino acid sequence is indicated over the nucleotide sequence. The same 3′ primer was used in the PCR amplification of both insertion mutants.

FIGURE 12 shows the SDS/PAGE profiles of the mutant enzymes purified to homogeneity. Lanes: 1, protein standards; 2, *Fok*I; 3, mutant *Fok*I with 4-codon insertion; and 4, mutant *FokI* with 7-codon insertion.

FIGURE 13 shows an analysis of the DNA sequence specificity of the mutant enzymes. The DNA substrates were digested in 10 mM Tris HCl, pH 8.0/50 mM NaCl/1 mM DTT/10mM $MgCl_2$ at 37°C for 2 hrs.

(A) Cleavage pattern of pTZ19R DNA substrate analyzed by 1% agarose gel electrophoresis. 2μg of pTZ19R DNA was used in each reaction. Lanes: 1, 1-kilobase (kb) ladder; 2, pTZ19R; 3, pTZ19R digested with *Fok*I; pTZ19R digested with mutant *Fok*I with 4-codon insertion; and 5, pTZ19R digested with mutant *FokI* with 7-codon insertion.

(B) Cleavage pattern of 256 bp DNA substrate containing a single *FokI* site analyzed by 1.5% agarose gel electrophoresis. 1μg of radiolabeled substrates (^{32}P-labeled on individual strands) was digested as described above. The agarose gel was stained with ethidium bromide and visualized under UV light. Lanes 2 to 6 correspond to the ^{32}P-labeled substrate in which the 5′-CATCC-3′ strand is ^{32}-P labeled. Lanes 7 to 11 correspond to the substrate in which the 5′-GGATG-3′ strand is ^{32}P-labeled. Lanes: 1, 1kb ladder; 2 and 7, ^{32}P-labeled 250 bp DNA substrates; 3 and 8, ^{32}-P labeled substrates cleaved with *Fok*I; 4 and 9, purified the laboratory wild-type *Fok*I; 5 and 10, mutant *Fok*I with 4-codon insertion; 6 and 11, mutant *Fok*I with 7-codon insertion.

(C) Autoradiograph of the agarose gel from above. Lanes: 2 to 11, same as in B.

FIGURE 14 shows an analysis of the distance of cleavage from the recognition site by *FokI* and the mutant enzymes. The unphosphorylated oligonucleotides were used for dideoxy DNA sequencing with pTZ19R as the template. The sequencing products (G, A, T, C) were electrophoresed on a 6% acrylamide gel containing 7M urea, and the gel dried. The products were then exposed to an x-ray film for 2 hrs. Cleavage products from the 100 bp and the 256 bp DNA substrates are shown in A and B, respectively. I

corresponds to substrates containing ^{32}P-label on the 5′-GGATG-3′ strand, and II corresponds to substrates containing ^{32}P-label on the 5′-CATCC-3′ strand. Lanes: 1, *Fok*I; 2, *Fok*I ; 3, mutant *Fok*I with 4-codon insertion; and 4, mutant *Fok*I with 7-codon insertion.

FIGURE 15 shows a map of the cleavage site(s) of *Fok*I and the mutant enzymes based on the 100 bp DNA substrate containing a single *Fok*I site: (A) wild-type *Fok*I; (B) mutant *Fok*I with 4-codon insertion; and (C) mutant *Fok*I with 7-codon insertion (see SEQ ID NO:40). The sites of cleavage are indicated by the arrows. Major cleavage sites are shown by larger arrows.

FIGURE 16 represents a diagram showing the orientation of the *Ubx* homeo domain with respect to the *Fok*I nuclease domain (F_N) in relation to the DNA substrate. The crystal structure of an *engrailed* homeo domain - DNA complex was reported by Kissinger et al. (Cell 63: 579-90 (1990)).

FIGURE 17 shows the construction of expression vectors of the *Ubx*-F_N hybrid enzyme. (A) Sequences of the 5′ and 3′ primers used to construct the hybrid gene, *Ubx*-F_N. The *Ubx* primers are flanked by *Pst*I and *Spe*I sites (see SEQ ID NO:41 and SEQ ID NO:42). The *Ubx*-F_N primers are flanked by *Nde*I and *Bam*HI sites (see SEQ ID NO:43 and SEQ ID NO:44). Start and stop codons are shown in boldface letters. (B) Structure of plasmids, pRRS *Ubx*-F_N and pET-15b *Ubx*-F_N. The PCR modified *Ubx* homeo box was substituted for the *Pst*I/*Spe*I fragment of pRRS*fokIR* to generate pRRS *Ubx*-F_N. The PCR-generated fragment using *Ubx*-F_N primers was inserted at the *Bam*HI/*Nde*I sites of pET-15b to form pET-15b *Ubx*-F_N.

FIGURE 18 represents SDS/PAGE profiles at each step in the purification of the *Ubx*-F_N hybrid

enzyme. Lanes: 1, protein standards; 2, crude extract from induced cells; 3, His-bind™ resin pool; 4, phosphocellulose pool; and 5, DEAE pool.

FIGURE 19 shows a characterization of the *Ubx-F_N* hybrid protein using the linearized pUC13 DNA substrates containing Ubx site(s). (A) pUC13 derived DNA substrates. □:30 bp insert containing the *Ubx* site, 5'-TTAATGGTT-3'. The number of tandem repeats of the 30 bp insert in these substrates are shown in brackets. The orientation of the *Ubx* site(s) are indicated by the arrows. (B) The DNA substrate (1 μg) was partially digested in buffer containing 20 mM Tris. HCl (pH 7.6), 75 mM KCl, 1 mM DTT, 50 μg/ml BSA, 10% glycerol, 100 mg/ml tRNA and 2 mM $MgCl_2$ at 31°C for 4-5 hrs. The products were analyzed by 1% agarose gel electrophoresis. The substrate was present in large excess compared to the *Ubx-F_N* hybrid protein (~100:1). The reaction condition was optimized to yield a single double-stranded cleavage per substrate molecule. The reaction proceeds to completion upon increasing the enzyme concentration or by digesting overnight at 31°C (data not shown). The two fragments, ~1.8 kb and ~0.95 kb, respectively, resulting from the binding of the hybrid enzyme at the newly inserted *Ubx* site of pUC13 and cleaving near this site, are indicated by the arrows.

FIGURE 20 shows an analysis of the distance of cleavage from the recognition site by *Ubx-F_N*. The cleavage products of the ^{32}P-labeled DNA substrate containing a single *Ubx* site by *Ubx-F_N* along with (G + A) Maxam-Gilbert sequencing reactions were separated by electrophoresis on a 6% polyacrylamide gel containing 6M urea, and the gel was dried and exposed to an x-ray film for 6 hrs. (A) corresponds to cleavage product(s) from a substrate containing ^{32}P-label on the 5'-TAAT-3'

strand (see SEQ ID NO:45). Lanes: 1, (G + A) sequencing reaction; and 2, *Ubx-F*$_N$. (B) corresponds to a substrate containing ^{32}P-label on the complementary strand, 5'-ATTA-3' (see SEQ ID NO:46). Lanes: 1, (G + A) sequencing reaction; 2, *Ubx-F*$_N$. (C) A map of the cleavage site(s) of *Ubx-F*$_N$ based on the DNA substrate containing a single *Ubx* site. The recognition site is shown by outline letters. The site(s) of cleavage are indicated by the arrows. The purine residues are indicated by * (see SEQ ID NO:47 and SEQ ID NO:48).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the identification and characterization of the functional domains of the *Fok*I restriction endonuclease. In the experiments resulting in the present invention, it was discovered that the *Fok*I restriction endonuclease is a two domain system, one domain of which possesses the sequence-specific recognition activity while the other domain contains the nuclease cleavage activity.

The *Fok*I restriction endonuclease recognizes the non-palindromic pentanucleotide 5'-GGATG-3'(SEQ ID NO:1):5'-CATCC-3'(SEQ ID NO:2) in duplex DNA and cleaves 9/13 nucleotides downstream from the recognition site. Since 10 base pairs are required for one turn of the DNA helix, the present inventor hypothesized that the enzyme would interact with one face of the DNA by binding at one point and cleave at another point on the next turn of the helix. This suggested the presence of two separate protein domains, one for sequence-specific recognition of DNA and one for endonuclease activity. The hypothesized two domain structure was shown to be the correct structure of the *Fok*I

endonuclease system by studies that resulted in the present invention.

Accordingly, in one embodiment, the present invention relates to a DNA segment which encodes the N-terminus of the *Fok*I restriction endonuclease (preferably, about the N-terminal 2/3's of the protein). This DNA segment encodes a protein which has the sequence-specific recognition activity of the endonuclease, that is, the encoded protein recognizes the non-palindromic pentanucleotide d-5'-GGATG-3'(SEQ ID NO:1):5'-CATCC-3'(SEQ ID NO:2) in duplex DNA. Preferably, the DNA segment of the present invention encodes amino acids 1-382 of the *Fok*I endonuclease.

In a further embodiment, the present invention relates to a DNA segment which encodes the C-terminus of the *Fok*I restriction endonuclease. The protein encoded by this DNA segment of the present invention has the nuclease cleavage activity of the *Fok*I restriction endonuclease. Preferably, the DNA segment of the present invention encodes amino acids 383-578 of the *Fok*I endonuclease. DNA segments of the present invention can be readily isolated from biological samples using methods known in the art, for example, gel electrophoresis, affinity chromatography, polymerase chain reaction (PCR), or a combination thereof. Further, the DNA segments of the present invention can be chemically synthesized using standard methods in the art.

The present invention also relates to the proteins encoded by the DNA segments of the present invention. Thus, in another embodiment, the present invention relates to a protein consisting essentially of the N-terminus of the *Fok*I endonuclease which retains the sequence-specific recognition activity of the enzyme. This protein of the present invention has a molecular weight of

about 41 kilodaltons as determined by SDS polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol.

In a further embodiment, the present invention relates to a protein consisting essentially of the C-terminus of the *Fok*I restriction endonuclease (preferably, the C-terminal 1/3 of the protein). The molecular weight of this protein is about 25 kilodaltons as determined by SDS/polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol.

The proteins of the present invention can be isolated or purified from a biological sample using methods known in the art. For example, the proteins can be obtained by isolating and cleaving the *Fok*I restriction endonuclease. Alternatively, the proteins of the present invention can be chemically synthesized or produced using recombinant DNA technology and purified.

The DNA segments of the present invention can be used to generate 'hybrid' restriction enzymes by linking other DNA binding protein domains with the nuclease or cleavage domain of *Fok*I. This can be achieved chemically as well as by recombinant DNA technology. Such chimeric hybrid enzymes have novel sequence specificity and are useful for physical mapping and sequencing of genomes of various species, such as, humans, mice and plants. For example, such enzymes would be suitable for use in mapping the human genome. These engineered hybrid endonucleases will also facilitate the manipulation of genomic DNA and provide valuable information about protein structure and protein design.

Such chimeric enzymes are also valuable research tools in recombinant DNA technology and molecular biology. Currently only 4-6 base pair cutters and a few 8 base pair cutters are available

commercially. (There are about 10 endonucleases which cut >6 base pairs that are available commercially.) By linking other DNA binding proteins to the nuclease domain of *Fok*I new enzymes can be generated that recognize more than 6 base pairs in DNA.

Accordingly, in a further embodiment, the present invention relates to a DNA construct and the hybrid restriction enzyme encoded therein. The DNA construct of the present invention comprises a first DNA segment encoding the nuclease domain of the *Fok*I restriction endonuclease, a second DNA segment encoding a sequence-specific recognition domain and a vector. The first DNA segment and the second DNA segment are operably linked to the vector so that expression of the segments can be effected thereby yielding a chimeric restriction enzyme. The construct can comprise regulatory elements such as promoters (for example, T7, *tac*, *trp* and lac UV5 promoters), transcriptional terminators or retroregulators (for example, stem loops). Host cells (procaryotes such as *E. coli*) can be transformed with the DNA constructs of the present invention and used for the production of chimeric restriction enzymes.

The hybrid enzymes of the present invention are comprised of the nuclease domain of *Fok*I linked to a recognition domain of another enzyme or DNA binding protein (such as, naturally occurring DNA binding proteins that recognize 6 base pairs). Suitable recognition domains include, but are not limited to, the recognition domains of zinc finger motifs; homeo domain motifs; POU domains (eukaryotic trnscription regulators, e.g., Pit1, Oct1, Oct2 and *unc*86); other DNA binding protein domains of *lambda* repressor, *lac* repressor, *cro*, gal4; DNA binding protein domains of oncogenes such

as *myc*, *jun*; and other naturally occurring sequence-specific DNA binding proteins that recognize >6 base pairs.

The hybrid restriction enzymes of the present invention can be produced by those skilled in the art using known methodology. For example, the enzymes can be chemically synthesized or produced using recombinant DNA technology well known in the art. The hybrid enzymes of the present invention can be produced by culturing host cells (such as, HB101, RR1, RB791 and MM294) containing the DNA construct of the present invention and isolating the protein. Further, the hybrid enzymes can be chemically synthesized, for example, by linking the nuclease domain of the *Fok*I to the recognition domain using common linkage methods known in the art, for example, using protein cross-linking agents such as EDC/NHS, DSP, etc.

One particular hybrid enzyme which can be created according to the present invention and, thus, an embodiment of the present invention is *Ubx*-F_N. The chimeric restriction endonuclease can be produced by linking the *Ubx* homeo domain to the cleavage domain (F_N) of *Fok*I. Subsequent to purification, the properties of the hybrid enzyme were analyzed.

While the *Fok*I restriction endonuclease was the enzyme studied in the following experiments, it is expected that other Type IIS endonucleases (such as, those listed in Table 2) will function using a similar two domain structure which one skilled in the art could readily determine based on the present invention.

Recently, *Sts*I, a heteroschizomer of *Fok*I has been isolated from *Streptococcus sanguis* (Kita et al., Nucleic Acids Research 20 (3)) 618, 1992). *Sts*I recognizes the same nonpalindromic

pentadeoxyribonucleotide 5'-GGATG-3':5'-CATCC-3' as *Fok*I but cleaves 10/14 nucleotides downstream of the recognition site. The *Sts*I *RM* system has been cloned and sequenced (Kita et al., Nucleic Acids Research 20 (16) 4167-72, 1992). Considerable amino acid sequence homology (~30%) has been detected between the endonucleases, *Fok*I and *Sts*I.

Another embodiment of the invention relates to the construction of two insertion mutants of *Fok*I endonuclease using the polymerase chain reaction (PCR). In particular, this embodiment includes a DNA construct comprising a first DNA segment encoding the catalytic domain of a Type IIS endonuclease which contains the cleavage activity of the Type IIS endonuclease, a second DNA segment encoding a sequence-specific recognition domain other than the recognition domain of the Type IIS endonuclease, and a third DNA segment comprising one or more codons. The third DNA segment is inserted between the first DNA segment and the second DNA segment. The construct also includes a vector. The Type IIS endonuclease is *Fok*I restriction endonuclease.

Suitable recognition domains include, but are not limited to, zinc finger motifs, homeo domain motifs, POU domains, DNA binding domains of repressors, DNA binding domains of oncogenes and naturally occurring sequence-specific DNA binding proteins that recognize >6 base pairs.

As noted above, the recognition domain of *Fok*I restriction endonuclease is at the amino terminus of *Fok*I endonuclease, whereas the cleavage domain is probably at the carboxyl terminal third of the molecule. It is likely that the domains are connected by a linker region, which defines the spacing between the recognition and the cleavage sites of the DNA substrate. This linker region of

*Fok*I is susceptible to cleavage by trypsin in the presence of a DNA substrate yielding a 41-kDa amino-terminal fragment (The DNA binding domain) and a 25-kDa carboxyl-terminal fragment (the cleavage domain). Secondary structure prediction of *Fok*I endonuclease based on its primary amino acid sequence supports this hypothesis (see Figure 10). The predicted structure reveals a long stretch of alpha helix region at the junction of the recognition and cleavage domains. This helix probably constitutes the linker which connects the two domains of the enzyme. Thus, it was thought that the cleavage distance of *Fok*I from the recognition site could be altered by changing the length of this spacer (the alpha helix). Since 3.6 amino acids are required to form one turn of the alpha helix, insertion of either four codons or seven codons in this region would extend the pre-existing helix in the native enzyme by one or two turns, respectively. Close examination of the amino acid sequence of this helix region revealed the presence of two KSEL repeats separated by amino acids EEK (Figure 10) (see SEQ ID NO:21). The segments KSEL (4 codons) (see SEQ ID NO:22) and KSELEEK (7 codons) (see SEQ ID NO:23) appeared to be good choices for insertion within this helix in order to extend it by one and two turns, respectively. (See Examples X and XI.) Thus, genetic engineering was utilized in order to create mutant enzymes.

In particular, the mutants are obtained by inserting one or more, and preferably four or seven, codons between the recognition and cleavage domains of *Fok*I. More specifically, the four or seven codons are inserted at nucleotide 1152 of the gene encoding the endonuclease. The mutants have the same DNA sequence specificity as the wild-type

enzyme. However, they cleave one nucleotide further away from the recognition site on both strands of the DNA substrates as compared to the wild-type enzyme.

Analysis of the cut sites of *Fok*I and the mutants, based on the cleavage of the 100 bp fragment, is summarized in Figure 15. Insertion of four (or seven) codons between the recognition and cleavage domains of *Fok*I is accompanied by an increase in the distance of cleavage from the recognition site. This information further supports the presence of two separate protein domains within the *Fok*I endonuclease: one for the sequence specific recognition and the other for the endonuclease activity. The two domains are connected by a linker region which defines the spacing between the recognition and the cleavage sites of the DNA substrate. The modular structure of the enzyme suggests it may be feasible to construct chimeric endonucleases of different sequence specificity by linking other DNA-binding proteins to the cleavage domain of the *Fok*I endonuclease.

In view of the above-information, another embodiment of the invention includes a procaryotic cell comprising a first DNA segment encoding the catalytic domain of a Type IIS endonuclease which contains the cleavage activity of the Type IIS endonuclease, a second DNA segment encoding a sequence-specific recognition domain other than the recognition domain of the Type IIS endonuclease, and a third DNA segment comprising one or more codons. The third DNA segment is inserted between the first DNA segment and the second DNA segment. The cell also includes a vector. Additionally, it should be noted that the first DNA segment, the second DNA segment, and the third DNA segment are operably

linked to the vector so that a single protein is produced. The third segment may consist essentially of four or seven codons.

The present invention also includes the protein produced by the procaryotic cell referred to directly above. In particular, the isolated protein consists essentially of the recognition domain of the FokI restriction endonuclease, the catalytic domain of the FokI restriction endonuclease, and amino acids encoded by the codons present in the third DNA segment.

The following non-limiting Examples are provided to describe the present invention in greater detail.

EXAMPLES

The following materials and methods were utilized in the isolation and characterization of the *Fok*I restriction endonuclease functional domains as exemplified hereinbelow.

Bacterial strains and plasmids

Recombinant plasmids were transformed into *E.coli* RB791 *i*^{q} cells which carry the *lac i*^{q} allele on the chromosome (Brent and Ptashne, PNAS USA, 78:4204-4208, 1981) or *E.coli* RR1 cells. Plasmid pACYC*fokIM* is a derivative of pACYC184 carrying the PCR-generated *fokIM* gene inserted into *NcoI* site. The plasmid expresses the *Fok*I methylase constitutively and was present in RB791 cells (or RR1 cells) whenever the *fokIR* gene was introduced on a separate compatible plasmid. The *Fok*I methylase modifies *Fok*I sites and provides protection against chromosomal cleavage. The construction of vectors pRRS and pCB are described elsewhere (Skoglund et al., Gene, 88:1-5, 1990).

Enzymes, biochemicals and oligos

Oligo primers for PCR were synthesized with an Applied Biosystem DNA synthesizer using cyanoethyl phosphoramidite chemistry and purified by reversed phase HPLC. Restriction enzymes were purchased from New England Biolabs. The DNA ligase IPTG were from Boehringer-Mannheim. PCR reagents were purchased as a Gene Amp Kit from Perkin-Elmer. Plasmid purification kit was from QIAGEN.

Restriction enzyme assays

Cells from a 5-ml sample of culture medium were harvested by centrifugation, resuspended in 0.5 ml sonication buffer [50 mM Tris.HCl (pH 8), 14mM 2-mercaptoethanol], and disrupted by sonication (3 x 5 seconds each) on ice. The cellular debris was centrifuged and the crude extract used in the enzyme assay. Reaction mixtures (10 μl) contained 10mM Tris.HCl (pH 8), 10 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 50 μg of BSA, 1 μg of plasmid pTZ19R (U.S. biochemicals) and 1μl of crude enzyme. Incubation was at 37°C for 15 min. tRNA (10 μg) was added to the reaction mixtures when necessary to inhibit non-specific nucleases. After digestion, 1 μl of dye solution (100 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol, 50% glycerol) was added, and the samples were electrophoresed on a 1% agarose gel. Bands were stained with 0.5 μg ethidium bromide/ml and visualized with 310-nm ultraviolet light.

SDS/PAGE

Proteins were prepared in sample buffer and electrophoresed in SDS (0.1%)-polyacrylamide (12%) gels as described by Laemmli (Laemmli, Nature, 222:680-685, 1970). Proteins were stained with coomassie blue.

Example I

Cloning of *Fok*I RM system

The *Fok*I system was cloned by selecting for the modification phenotype. *Flavobacterium okeanokoites* strain DNA was isolated by the method described by Caserta et al. (Caserta et al., J. Biol. Chem., 262:4770-4777, 1987). Several *Flavobacterium okeanokoites* genome libraries were constructed in plasmids pBR322 and pUC13 using the cloning enzymes *PstI, Bam*HI *and Bgl*II. Plasmid library DNA (10 µg) was digested with 100 units of *Fok*I endonuclease to select for plasmids expressing *fokIM*+ phenotype.

Surviving plasmids were transformed into RR1 cells and transformants were selected on plates containing appropriate antibiotic. After two rounds of biochemical enrichment, several plasmids expressing the *fokIM*+ phenotype from these libraries were identified. Plasmids from these clones were totally resistant to digestion by *Fok*I.

Among eight transformants that were analyzed from the *F. okeanokoites* pBR322 *PstI* library, two appeared to carry the *fokIM* gene and plasmids from these contained a 5.5 kb *PstI* fragment. Among eight transformants that were picked from *F. okeanokoites* pBR322 *Bam*HI library, two appeared to carry the *fokIM* gene and their plasmids contained ~ 18 kb *Bam*HI fragment. Among eight transformants that were analyzed from the *F. okeanokoites* genome *Bgl*II library in pUC13, six appeared to carry the *fokIM* gene. Three of these clones had a 8 kb *Bgl*II insert while the rest contained a 16 kb *Bgl*II fragment.

Plating efficiency of phage λ on these clones suggested that they also carried the *fokIR* gene. The clones with the 8-kb *Bgl*II insert

appeared to be most resistant to phage infection. Furthermore, the *Fok*I endonuclease activity was detected in the crude extract of this clone after partial purification on a phosphocellulose column. The plasmid, pUC*fokIRM* from this clone was chosen for further characterization.

The 5.5 kb *PstI* fragment was transferred to M13 phages and the nucleotide sequences of parts of this insert determined using Sanger's sequencing method (Sanger et al., PNAS USA, 74:5463-5467, 1977). The complete nucleotide sequence of the *Fok*I RM system has been published by other laboratories (Looney et al., Gene, 80:193-208, 1989; Kita et al., Nucleic Acid Res., 17:8741-8753, 1989; Kita et al., J. Biol. Chem. 264:5751-5756, 1989).

Example II

Construction of an efficient overproducer clone of *Fok*I endonuclease using polymerase chain reaction.

The PCR technique was used to alter transcriptional and translational signals surrounding the *fokIR* gene so as to achieve overexpression in *E.coli* (Skoglund et al., Gene, 88:1-5, 1990). The ribosome-binding site preceding the *fokIR* and *fokIM* genes were altered to match the consensus *E. coli* signal.

In the PCR reaction, plasmid pUC*fokIRM* DNA linearized with *Bam*HI was used as the template. PCR reactions (100 μl) contained 0.25 nmol of each primer, 50 μM of each dNTP, 10 mM Tris.HCl (pH 8.3 at 25°C), 50 mM KCl, 1.5 mM $MgCl_2$ 0.01% (W/V) gelatin, 1 ng of template DNA, 5 units of *Taq* DNA polymerase. The oligo primes used for the amplification of the *fokIR* and *fokIM* genes are shown in Figure 1. Reaction mixtures (ran in

quadruplicate) were overlayed with mineral oil and reactions were carried out using Perkin-Elmer-Cetus Thermal Cycler.

Initial template denaturation was programmed for 2 min. Thereafter, the cycle profile was programmed as follows: 2 min at 37°C (annealing), 5 min at 72°C (extension), and 1 min at 94°C (denaturation). This profile was repeated for 25 cycles and the final 72°C extension was increased to 10 min. The aqueous layers of the reaction mixtures were pooled and extracted once with 1:1 phenol/chloroform and twice with chloroform. The DNA was ethanol-precipitated and resuspended in 20 μl TE buffer [10 mM Tris.HCl, (pH 7.5), 1 mM EDTA]. The DNA was then cleaved with appropriate restriction enzymes to generate cohesive ends and gel-purified.

The construction of an over-producer clone was done in two steps. First, the PCR-generated DNA containing the *fokIM* gene was digested with *NcoI* and gel purified. It was then ligated into *NcoI*-cleaved and dephosphorylated pACYC184 and the recombinant DNA transfected into *E.coli* RB791 i^q or RR1 cells made competent as described by Maniatis et al (Maniatis et al., <u>Molecular Cloning. A laboratory manual Cold Spring Harbor Laboratory, Cold Spring Harbor, NY</u>, 1982). After Tc selection, several clones were picked and plasmid DNA was examined by restriction analysis for the presence of *fokIM* gene fragment in correct orientation to the chloramphenicol promoter of the vector (see figure 2). This plasmid expresses *Fok*I methylase constitutively, and this protects the host from chromosomal cleavage when the *fokIR* gene is introduced into the host on a compatible plasmid. The plasmid DNA from these clones are therefore resistant to *Fok*I digestion.

Second, the PCR-generated *fokIR* fragment was ligated into *Bam*HI-cleaved and dephosphorylated high expression vectors pRRS or pCB. pRRS possesses a *lac* UV5 promoter and pCB containing the strong *tac* promoter. In addition, these vectors contain the positive retroregulator stem-loop sequence derived from the crystal protein-encoding gene of *Bacillus Thuringiensis* downstream of the inserted *fokIR* gene. The recombinant DNA was transfected into competent *E.coli* RB791 i^q [pACYC*fokIM*] or RR1[pACYC*fokIM*]cells. After Tc and Ap antibiotic selection, several clones were picked and plasmid DNA was examined by restriction analysis for *fokIR* gene fragment in correct orientation for expression from the vector promoters. These constructs were then examined for enzyme production.

To produce the enzyme, plasmid-containing RB791 i^q or RR1 cells were grown at 37°C with shaking in 2x concentrated TY medium [1.6% tryptone, 1% yeast extract, 0.5% NaCl (pH 7.2)] supplemented with 20 μg Tc/ml (except for the pUC*fokIRM* plasmid) and 50 μg Ap/ml. IPTG was added to a concentration of 1 mM when the cell density reached $O.D._{600}$ = 0.8. The cells were incubated overnight (12 hr) with shaking. As is shown in Figure 2, both constructs yield *Fok*I to a level of 5-8% of the total cellular protein.

Example III

Purification of *FoK*I endonuclease

A simple three-step purification procedure was used to obtain electrophoretically homogeneous *Fok*I endonuclease. RR1 [pACYC*fokIM*, pRRS*fokIR*] were grown in 6L of 2 x TY containing 20μg Tc/ml and 50 μg/Ap ml at 37°C to A_{600} = 0.8. and then induced overnight with 1 mM IPTG. The cells were harvested by centrifugation and then resuspended in 250 ml of buffer A [10 mM Tris.phosphate (pH 8.0), 7 mM 2-

mercaptoethanol, 1 mM EDTA, 10% glycerol] containing 50 mM NaCl.

The cells were disrupted at maximum intensity on a Branson Sonicator for 1 hr at 4°C. The sonicated cells were centrifuged at 12,000 g for 2 hr at 4°C. The supernatant was then diluted to 1L with buffer A containing 50 mM NaCl. The supernatant was loaded onto a 10 ml phosphocellulose (Whatman) column pre-equilibrated with buffer A containing 50 mM NaCl. The column was washed with 50 ml of loading buffer and the protein was eluted with a 80-ml total gradient of 0.05M to 0.5M NaCl in buffer A. The fractions were monitored by A_{280} absorption and analyzed by electrophoresis on SDS (0.1%)-polyacrylamide (12%) gels (Laemmli, Nature, 222:680-685, 1970). Proteins were stained with coomassie blue.

Restriction endonuclease activity of the fractions were assayed using pTZ19R as substrate. The fractions containing *Fok*I were pooled and fractionated with ammonium sulfate. The 50-70% ammonium sulfate fraction contained the *Fok*I endonuclease. The precipitate was resuspended in 50 ml of buffer A containing 25 mM NaCl and loaded onto a DEAE column. *Fok*I does not bind to DEAE while many contaminating proteins do. The flow-through was concentrated on a phosphocellulose column. Further purification was achieved using gel filtration (AcA 44) column. The *Fok*I was purified to electrophoretic homogeneity using this procedure.

SDS (0.1%) polyacrylamide (12%) gel electrophoresis profiles of protein species present at each stage of purification are shown in Figure 3. The sequence of the first ten amino acids of the purified enzyme was determined by protein sequencing. The determined sequence was the same as that predicted from the nucleotide sequence.

Crystals of this purified enzyme have also been grown using PEG 4000 as the precipitant. *Fok*I endonuclease was purified further using AcA44 gel filtration column.

Example IV

Analysis of *FokIR* endonuclease by trypsin cleavage in the presence of DNA substrate.

Trypsin is a serine protease and it cleaves at the C-terminal side of lysine and arginine residues. This is a very useful enzyme to study the domain structure of proteins and enzymes. Trypsin digestion of *Fok*I in the presence of its substrate, d-5'-CCTCTGGATGCTCTC-3'(SEQ ID NO:10): 5'-GAGAGCATCCAGAGG-3' (SEQ ID NO:11) was carried out with an oligonucleotide duplex to *Fok*I molar ratio of 2.5:1. *Fok*I (200 μg) was incubated with the oligonucleotide duplex in a volume 180 μl containing 10 mM Tris.HCl, 50 mM NaCl, 10% glycerol and 10 mM $MgCl_2$ at RT for 1 hr. Trypsin (20 μl, 0.2 mg/ml) was added to the mixture. Aliquots (28 μl) from the reaction mixture were removed at different time intervals and quenched with excess trypsin inhibitor, antipain. The tryptic fragments were purified by reversed-phase HPLC and their N-terminus sequence determined using an automatic protein sequenator from Applied Biosystems.

The time course of trypsin digestion of *Fok*I endonuclease in the presence of 2.5 molar excess of oligonucleotide substrate and 10 mM $MgCl_2$ is shown in Figure 4. At the 2.5 min time point only two major fragments other than the intact *Fok*I were present, a 41 kDa fragment and a 25 kDa fragment. Upon further trypsin digestion, the 41 kDa fragment degraded into a 30 kDa fragment and 11 kDA fragment. The 25 kDa fragment appeared to be

resistant to any further trypsin digestion. This fragment appeared to be less stable if the trypsin digestion of *Fok*I - oligo complex was carried out in the absence of $MgCl_2$.

Only three major fragments (30 kDa, 25 kDa and 11 kDa) were present at the 160 min time point. Each of these fragments (41 kDa, 30 kDa, 25 kDa and 11 kDa) was purified by reversed-phase HPLC and their N-terminal amino acid sequence were determined (Table I). By comparing these N-terminal sequences to the predicted sequence of *Fok*I, the 41 kDa and 25 kDa fragments were identified as N-terminal and C-terminal fragments, respectively. In addition, the 30 kDa fragment was N-terminal.

Example V

Isolation of DNA binding tryptic fragments of *Fok*I endonuclease using oligo dT-cellulose affinity column.

The DNA binding properties of the tryptic fragments were analyzed using an oligo dT-cellulose column. *Fok*I (160 μg) was incubated with the 2.5 molar excess oligonucleotide duplex [d-5'-CCTCTGGATGCTCTC$(A)_{15}$-3' (SEQ ID NO:14): 5'GAGAGCATCCAGAGG$(A)_{15}$-3' (SEQ ID NO:15)] in a volume of 90 μl containing 10 mM Tris.HCl (pH 8), 50 mM NaCl, 10% glycerol and 10 mM $MgCl_2$ at RT for 1 hr. Trypsin (10 μl, 0.2 mg/ml) was added to the solution to initiate digestion. The ratio of trypsin to *Fok*I (by weight) was 1:80. Digestion was carried out for 10 min to obtain predominantly 41 kDa N-terminal fragment and 25 kDa C-terminal fragments in the reaction mixture. The reaction was quenched with large excess of antipain (10 μg) and diluted in loading buffer [10 mM.Tris HCl (pH 8.0), 1 mM EDTA and 100 mM $MgCl_2$] to a final volume of 400 μl.

The solution was loaded onto a oligo dT-cellulose column (0.5 ml, Sigma, catalog #0-7751) pre-equilibrated with the loading buffer. The breakthrough was passed over the oligo dT-cellulose column six times. The column was washed with 5 ml of loading buffer and then eluted twice with 0.4 ml of 10 mM Tris.HCl (pH 8.0), 1 mM EDTA. These fractions contained the tryptic fragments that were bound to the oligonucleotide DNA substrate. The tryptic fragment bound to the oligo dT-cellulose column was analyzed by SDS-polyacrylamide gel electrophoresis.

In a separate reaction, the trypsin digestion was carried out for 160 min to obtain predominantly the 30 kDa, 25 kDa and 11 kDa fragments in the reaction mixture.

Trypsin digestion of *Fok*I endonuclease for 10 min yielded the 41 kDa N-terminal fragment and 25 kDa C-terminal fragments as the predominant species in the reaction mixture (Figure 5, Lane 3). When this mixture was passed over the oligo dT-cellulose column, only the 41 kDa N-terminal fragment is retained by the column suggesting that the DNA binding property of *Fok*I endonuclease is in the N-terminal 2/3's of the enzyme. The 25 kDa fragment is not retained by the oligo dT-cellulose column.

Trypsin digestion of *Fok*I - oligo complex for 160 min yielded predominantly the 30 kDa, 25 kDa and 11 kDa fragments (Figure 5, Lane 5). When this reaction mixture was passed over oligo dT-cellulose column, only the 30 kDa and 11 kDa fragments were retained. It appears these species together bind DNA and they arise from further degradation of 41 kDa N-terminal fragment. The 25 kDa fragment was not retained by oligo dT-cellulose column. It also did not bind to DEAE and thus could be purified by

passage through a DEAE column and recovering it in the breakthrough volume.

*Fok*I (390 μg) was incubated with 2.5 molar excess of oligonucleotide duplex [d-5'-CTCTGGATGCTCTC-3'(SEQ ID NO:10) :5'-GAGAGCATCCAGAGG-3'(SEQ ID NO:11)] in a total volume of 170 μl containing 10 mM Tris.HCl (pH 8), 50 mM NaCl and 10% glycerol at RT for 1 hr. Digestion with trypsin (30 μl; 0.2 mg/ml) in the absence of $MgCl_2$ was for 10 min at RT to maximize the yield of the 41 kDa N-terminal fragment. The reaction was quenched with excess antipain (200 μl). The tryptic digest was passed through a DEAE column. The 25 kDa of C-terminal fragment was recovered in the breakthrough volume. All the other tryptic fragments (41 kDa, 30 kDa and 11 kDa) were retained by the column and were eluted with 0.5M NaCl buffer (3 x 200 μl). In a separate experiment, the trypsin digestion of *Fok*I -oligo complex was done in presence of 10 mM $MgCl_2$ at RT for 60 min to maximize the yield of 30 kDa and 11 kDa fragments. This purified fragment cleaved non-specifically both unmethylated DNA substrate (pTZ19R; Figure 6) and methylated DNA substrate (pACYC*fokIM*) in the presence of $MgCl_2$. These products are small, indicating that it is relatively non-specific in cleavage. The products were dephosphorylated using calf intestinal phosphatase and rephosphorylated using polynucleotide kinase and [γ-^{32}P] ATP. The ^{32}P-labeled products were digested to mononucleotides using DNase I and snake venom phosphodiesterase. Analysis of the mononucleotides by PEI-cellulose chromatography indicates that the 25 kDa fragment cleaved preferentially phosphodiester bonds 5' to G>A>>T~C. The 25 kDa C-terminal fragment thus constitutes the cleavage domain of *Fok*I endonuclease.

The 41 kDa N-terminal fragment - oligo complex was purified by agarose gel electrophoresis. *Fok*I endonuclease (200 μg) was incubated with 2.5 molar excess of oligonucleotide duplex, [d-5' - CCTCTGGATGCTCTC-3'(SEQ ID NO: 10): 5'-GAGAGCATCCAGAGG-3'(SEQ ID NO:11)] in a volume of 180 μl containing 10 mM Tris.HCl (pH 8.0), 50 mM NaCl and 10% glycerol at RT for 1 hr. Tracer amounts of ^{32}P-labeled oligonucleotide duplex was incorporated into the complex to monitor it during gel electrophoresis. Digestion with trypsin (20 μl; 0.2 mg/ml) was for 12 min at RT to maximize the yield of the 41 kDa N-terminal fragment. The reaction was quenched with excess antipain. The 41 kDa N-terminal fragment - oligo complex was purified by agarose gel electrophoresis. The band corresponding to the complex was excised and recovered by electroelution in a dialysis bag (~ 600 μl). Analysis of the complex by SDS-PAGE revealed 41 kDa N-terminal fragment to be the major component. The 30 kDa N-terminal fragment and the 11 kDa C-terminal fragment were present as minor components. These together appeared to bind DNA and co-migrate with the 41 kDa N-terminal fragment-oligo complex.

The binding specificity of the 41 KDa N-terminal fragment was determined using gel mobility shift assays.

Example VI

Gel Mobility shift assays

The specific oligos (d-5'-CCTCTGGATGCTCTC-3'(SEQ ID NO:10) and d-5'-GAGAGCATCCAGAGG-3' (SEQ ID NO:11)) were 5'-^{32}P-labeled in a reaction mixture of 25 μl containing 40 mM Tris.HCl(pH7.5), 20mM $MgCl_2$,50 mM NaCl, 10 mM DTT, 10 units of T4 polynucleotide kinase (from New England Biolabs) and 20 μCi[γ-^{32}P]

ATP (3000 Ci/mmol). The mixture was incubated at 37°C for 30 min. The kinase was inactivated by heating the reaction mixture to 70°C for 15 min. After addition of 200 μl of water, the solution was passed through Sephadex G-25 (Superfine) column (Pharmacia) to remove the unreacted [γ-^{32}P] ATP. The final concentration of labeled single-strand oligos were 27 μM.

The single-strands were then annealed to form the duplex in 10 mM Tris.HCl (pH 8.0), 50 mM NaCl to a concentration of 12 μM. 1 μl of the solution contained ~ 12 picomoles of oligo duplex and ~ 50 x 10^3cpm. The non-specific oligos (d-5'-TAATTGATTCTTAA-3'(SEQ ID NO:12) and d-5'-ATTAAGAATCAATT-3'(SEQ ID NO:13)) were labeled with [γ-^{32}P]ATP and polynucleotide kinase as described herein. The single-stranded oligos were annealed to yield the duplex at a concentration of 12μM. 1 μl of the solution contained ~ 12 picomoles of oligo duplex and ~ 25 x 10^3cpm. The non-specific oligos (d-5'-TAATTGATTCTTAA-3'(SEQ ID NO:12) and d-5'-ATTAAGAATCAATT-3'(SEQ ID NO:13)) were labeled with [γ-^{32}P] ATP and polynucleotide Kinase as described herein. The single-strand oligos were annealed to yield the duplex at a concentration of 12μM. 1 μl of the solution contained 42 picomdes of oligo duplex and ~25x10^3 cpm.

10 μl of 41 kDa N-terminal fragment-oligo complex (~ 2 pmoles) in 10 mM Tris.HCl, 50 mM NaCl and 10 mM $MgCl_2$ was incubated with 1 μl of ^{32}P-labeled specific oligonucleotide duplex (or ^{32}P-labeled non-specific oligonucleotide duplex) at 37°C for 30 min and 120 min respectively. 5 μl of 75% glycerol was added to each sample and loaded on a 8% nondenaturing polyacrylamide gel. Electrophoresis was at 300 volts in TBE buffer until bromophenol

blue moved ~ 6 cm from the top of the gel. The gel was dried and autoradiographed.

The complex readily exchanged ^{32}P-labeled specific oligonucleotide duplex that contained the *Fok*I recognition site as seen from the gel mobility shift assays (Figure 7). It did not, however, exchange the ^{32}P-labeled non-specific oligonucleotide duplex that did not contain the *Fok*I recognition site. These results indicate that all the information necessary for sequence-specific recognition of DNA are encoded within the 41 kDa N-terminal fragment of *Fok*I.

Example VII
Analysis of *Fok*I by trypsin cleavage in the absence of DNA substrate.

A time course of trypsin digestion of *Fok*I endonuclease in the absence of the DNA substrate is shown in Figure 8. Initially, *Fok*I cleaved into a 58 kDa fragment and a 8 kDa fragment. The 58 kDa fragment did not bind DNA substrates and is not retained by the oligo dT-cellulose column. On further digestion, the 58 kDa fragment degraded into several intermediate tryptic fragments. However, the complete trypsin digestion yielded only 25 kDa fragments (appears as two overlapping bands).

Each of these species (58 kDa, 25 kDa and 8 kDa) were purified by reversed phase HPLC and their amino terminal amino acid sequence determined (Table I). Comparison of the N-terminal sequences to the predicted *Fok*I sequence revealed that the 8 kDa fragment to be N-terminal and the 58 kDa fragment to be C-terminal. This further supports the conclusion that N-terminus of *Fok*I is responsible for the recognition domain. Sequencing the N-terminus of the 25 kDa fragments revealed the presence of two different components. A time course

of trypsin digestion of *Fok*I endonuclease in a the presence of a non-specific DNA substrate yielded a profile similar to the one obtained when trypsin digestion of *Fok*I is carried out in absence of any DNA substrate.

Example VIII

Cleavage specificity of the 25 kDa C-terminal tryptic fragment of FokI

The 25 kDa C-terminal tryptic fragment of *Fok*I cleaved pTZ19R to small products indicating non-specific cleavage. The degradation products were dephosphorylated by calf intestinal phosphatase and ^{32}P-labeled with the polynucleotide kinase and [γ-^{32}P]ATP. The excess label was removed using a Sephadex G-25 (Superfine) column. The labeled products were then digested with 1 unit of pancreatic DNase I (Boehringer-Mannheim) in buffer containing 50 mM Tris.HCl(pH7.6), 10mM $MgCl_2$ at 37°C for 1 hr. Then, 0.02 units of snake venom phosphodiesterase was added to the reaction mixture and digested at 37°C for 1 hr.

Example IX

Functional domains in *Fok*I restriction endonuclease.

Analysis of functional domains of *Fok*I (in the presence and absence of substrates) using trypsin was summarized in Figure 9. Binding of DNA substrate by *Fok*I was accompanied by alteration in the structure of the enzyme. This study supports that presence of two separate protein domains within this enzyme: one for sequence-specific recognition and the other for endonuclease activity. The results indicate that the recognition domain is at the N-terminus of the *Fok*I endonuclease, while the

cleavage domain is probably in the C-terminus third of the molecule.

Examples Relating to Construction of Insertion Mutants (X-XIV)

The complete nucleotide sequence of the *Fok*I *RM* system has been published by various laboratories (Looney et al., Gene 80: 193-208, 1989 & Kita et al., J. Biol.Chem. 264: 5751-56, 1989). Experimental protocols for PCR are described, for example, in Skoglund et al., Gene 88:1-5, 1990 and in Bassing et al., Gene 113:83-88, 1992. The procedures for cell growth and purification of the mutant enzymes are similar to the ones used for the wild-type *Fok*I (Li et al., Proc. Nat'l. Acad. Sci. USA 89:4275-79, 1992). Additional steps which include Sephadex G-75 gel filtration and Heparin-Sepharose CL-6B column chromatography were necessary to purify the mutant enzymes to homogeneity.

Example X

Mutagensis of *Spe*I Site at Nucleotide 162 within the *fokIR* Gene

The two step PCR technique used to mutagenize one of the *Spe*I sites within the *fokIR* gene is described in Landt et al., Gene 96: 125-28, 1990. The three synthetic primers for this protocol include: 1) the mutagenic primer (5'-TCATAA TAGCAACTAATTCTTTTTGGATCTT-3') (see SEQ ID NO:24) containing one base mismatch within the *Spe*I site; 2) the other primers each of which are flanked by restriction sites *Cla*I (5'-CCATCGATATAGCCTTTTTTATT-3') (see SEQ ID NO:25) and *XbaI* (5'-GCTCTAGAGGATCCGGAGGT-3') (see SEQ ID NO:26), respectively. An intermediate fragment was amplified using the *Xba*I primer and the mutagenic primer during the first step. The *Cla*I primer was

then added to the intermediate for the second step PCR. The final 0.3 kb PCR product was digested with *Xba*I/*Cla*I to generate cohesive ends and gel-purified. The expression vector (pRRS*fokIR*) was cleaved with *Xba*I/*Cla*I. The large 4.2 kb fragment was then gel-purified and ligated to the PCR fragment. The recombinant DNA was transfected into competent *E. coli* RR1[pACYC*fokIM*] cells. After tetracycline and ampicillin antibiotic selection several clones were picked, and their plasmid DNA was examined by restriction analysis. The *Spe*I site mutation was confirmed by sequencing the plasmid DNA using Sanger's sequencing method (Sanger et al. Proc. Natl. Acad. Sci. USA 74: 5463-67, 1977).

Example XI
Construction of four (or seven) codon Insertion Mutants

The PCR-generated DNA containing a four (or seven) codon insertion was digested with a *Spe*I/*Xma*I and gel-purified. The plasmid, pRRS*fokIR* from Example X was cleaved with *Spe*I/*Xma*I, and the large 3.9 kb fragment was gel-purified and ligated to the PCR product. The recombinant DNA was transfected into competent RR1[pACYC*fokIM*] cells, and the desired clones identified as described in Example X. The plasmids from these clones were isolated and sequenced to confirm the presence of the four (or seven) codon insertion within the *fokIR* gene.

In particular, the construction of the mutants was performed as follows: (1) There are two *Spe*I sites at nucleotides 162 and 1152, respectively, within the *fokIR* gene sequence. The site at 1152 is located near the trypsin cleavage site of *Fok*I that separates the recognition and cleavage domains. In order to insert the four (or

seven) codons around this region, the other *Spe*I site at 162 was mutagenized using a two step PCR technique (Landt et al. Gene 96:125-28, 1990). Introduction of this *Spe*I site mutation in the *fokIR* gene does not affect the expression levels of the overproducer clones. (2) The insertion of four (or seven) codons was achieved using the PCR technique. The mutagenic primers used in the PCR amplification are shown in Figure 11. Each primer has a 21 bp complementary sequence to the *fokIR* gene. The 5′ end of these primers are flanked by *Spe*I sites. The codons for KSEL and KSELEEK repeats are incorporated between the *Spe*I site and the 21 bp complement. Degenerate codons were used in these repeats to circumvent potential problems during PCR amplification. The other primer is complementary to the 3′ end of the *fokIR* gene and is flanked by a *XmaI* site. The PCR-generated 0.6 kb fragments containing the four (or seven) codon inserts digested with *SpeI/XmaI* and gel-purified. These fragments were substituted into the high expression vector pRRS*fokIR* to generate the mutants. Several clones of each mutant identified and their DNA sequence confirmed by Sanger's dideoxy chain termination methoa (Sanger et al. Proc. Natl. Acad. Sci. USA 74.5463-67 1977).

Upon induction with 1 mM isopropyl ß-D-thiogalactoside (IPTG), the expression of mutant enzymes in these clones became most prominent at 3 hrs as determined by SDS/PAGE. This was further supported by the assays for the enzyme activity. The levels of expression of the mutant enzymes in these clones were much lower compared to the wild-type *Fok*I. IPTG induction for longer times resulted in lower enzyme levels indicating that the mutant enzymes were actively degraded within these clones. This suggests that the insertion of four (or seven)

codons between the recognition and cleavage domains of *FokI* destabilizes the protein conformation making them more susceptible to degradation within the cells. SDS/PAGE profiles of the mutant enzymes are shown in Figure 12.

Example XII
Preparation of DNA Substrates with a Single *Fok*I Site

Two substrates, each containing a single *Fok*I recognition site, were prepared by PCR using pTZ19R as the template. Oligonucleotide primers, 5′-CGCAGTGTTATCACTCAT-3′ and 5′-CTTGGTTGAGTACTCACC-3′ (see SEQ ID NO:27 and SEQ ID NO:28, respectively), were used to synthesize the 100 bp fragment. Primers, 5′-ACCGAGCTCGAATTCACT-3′ and 5′-GATTTCGGCCTATTGGTT-3′ (see SEQ ID NO:29 and SEQ ID NO:30, respectively), were used to prepare the 256 bp fragment. Individual strands within these substrates were radiolabled by using the corresponding ^{32}P-labeled phosphorylated primers during PCR. The products were purified from low-melting agarose gel, ethanol precipitated and resuspended in TE buffer.

Example XIII
Analysis of the Sequence Specificity of the Mutant Enzymes

The agarose gel electrophoretic profile of the cleavage products of pTZ19R DNA by *Fok*I and the mutants are shown in Figure 13A. They are very similar suggesting that insertion of four (or seven) codons in the linker region between the recognition and cleavage domains does <u>not</u> alter its DNA sequence specificity. This was further confirmed by using ^{32}P-labeled DNA substrates (100 bp and 256 bp) each containing a single *Fok*I site. Substrates

containing individual strands labeled with ^{32}P were prepared as described in Example XII. *Fok*I cleaves the 256 bp substrate into two fragments, 180 bp and 72 bp, respectively (Figure 13B). The length of the fragments was calculated from the ^{32}P-labeled 5′ end of each strand. The autoradiograph of the agarose gel is shown in Figure 13C. Depending on which strand carries the ^{32}P-label in the substrate, either 72 bp fragment or 180 bp fragment appears as a band in the autoradiograph. The mutant enzymes reveal identical agarose gel profiles and autoradiograph. Therefore, insertion of four (or seven) codons between the recognition and cleavage domains does not alter the DNA recognition mechanism of *Fok*I endonuclease.

Example XIV

Analysis of the Cleavage Distances from the Recognition Site by the Mutant Enzymes

To determine the distance of cleavage by the mutant enzymes, their cleavage products of the ^{32}P-labeled substrates were analyzed by PAGE (Figure 14). The digests were analyzed alongside the sequencing reactions of pTZ19R performed with the same primers used in PCR to synthesize these substrates. The cleavage pattern of the 100 bp fragment by *Fok*I and the mutants are shown in Figure 14A. The cut sites are shifted from the recognition site on both strands of the substrates in the case of the mutants, as compared to the wild-type enzyme. The small observable shifts between the sequencing gel and the cleavage products are due to the unphosphorylated primers that were used in the sequencing reactions.

On the 5′-GGATG-3′ strand, both mutants cut the DNA 10 nucleotides away from the site while on the 5′-CATCC-3′ strand they cut 14 nucleotides

away from the recognition site. These appear to be the major cut sites for both the mutants. A small amount of cleavage similar to the wild-type enzyme was is also observed.

The cleavage pattern of the 256 bp fragment is shown in Figure 14B. The pattern of cleavage is shown in Figure 14B. The pattern of cleavage is similar to the 100 bp fragment. Some cleavage is seen 15 nucleotides away from the recognition site on the 5'-CATCC-3' strand in the case of the mutants. The multiple cut sites for the mutant enzymes could be attributed to the presence of different conformations in these proteins. Or due to the increased flexibility of the spacer region between the two domains. Depending on the DNA substrate, some variation in the intensity of cleavage at these sites was observed. This may be due to the nucleotide sequence around these cut sites. Naturally occurring Type IIS enzymes with multiple cut sites have been reported (Szybalski et al., Gene 100:13-26, 1991).

Examples Relating to Construction of the Hybrid Enzyme *Ubx-F_N* (XV-XVII)

As noted above, the complete nucleotide sequence of the *Fok*I restriction-modification system has been published by other laboratories (Kita et al., J. Biol Chem. 264:5751-56 (1989); Looney et al., Gene 80:193-208 (1989)). Experimental protocols for PCR are described elsewhere (Skoglund et al., Gene 88:1-5 (1990)). The procedures for cell growth and purification of proteins using His-bind™ resin is as outlined in Novagen pET system manual. Additional steps, which include phosphocellulose and DEAE column chromatography, were necessary to purify the hybrid protein, *Ubx-F_N*

to near homogeneity. The protocol for SDS/PAGE is as described by Laemmli (Nature 222:680-685 (1970)).

Preparation of pUC13 derived substrates:

pUC13 derived DNA substrates were prepared by blunt-end ligation of *Sma*I-cleaved pUC13 plasmid with ten-fold excess of a 30 bp insert containing a known *Ubx* site, 5'-TTAATGGTT-3'. Several clones were picked and their plasmid DNA were analyzed for the presence of 30 bp inserts. Clones containing pUC13(1), pUC13(2) or pUC13(3), each with 1, 2 and 3 inserts respectively, were identified. Their DNA sequences were confirmed by Sanger's dideoxy sequencing method (Proc. Natl. Acad. Sci. USA 74:5463-67 (1977).

Preparation of DNA substrates with a single *Ubx* site:

The polylinker region of pUC13(1) which has a single 30 bp insert was excised using *Eco*RI/*Hin*dIII and gel-purified. Individual stands of his substrate were radiolabeled by using ^{32}P-dATP or ^{32}P-dCTP and filling in the sticky ends of the fragment with Klenow enzyme. The products were purified from low-melting agarose gel, ethanol-precipitated, and resuspended in the buffer (10 mM Tris.HCl/1 mM EDTA, pH 8.0).

Example XV

Construction of the Clone Producing the Hybrid Enzyme, *Ubx*-F_N Using PCR

The homeo domain of *Ubx*, a 61 amino acid protein sequence encoded by the homeobox of *Ubx* is a sequence-specific DNA-binding domain with a structure related to helix-turn-helix motifs found in bacterial DNA-binding proteins (Hayashi et al., Cell 63:883-94 (1992); Wolberger et al., Cell

67:517-28 (1991). The *Ubx* homeo domain recognizes the 9 bp consensus DNA sites, 5′-TTAAT (G/T) (G/A) CC-3′ (Ekker et al., The EMBO Journal 10:1179-86 (1991); Ekker et al., The EMBO Journal 11:4059-4702 (1992)). The present inventors used the PCR technique to link the *Ubx* homeo domain to the cleavage domain (F_N) of *Fok*I and to express the *Ubx*-F_N enzyme in *E. coli*. A schematic representation of the engineered *Ubx*-F_N hybrid protein is shown in Fig. 16. The oligonucleotide primers used to construct the hybrid gene is shown in Fig. 17A.

Construction of the clone expressing the hybrid protein was done as follows: First, the PCR-generated *Ubx* homeo box was digested with *Pst*I/*Spe*I and gel-purified. This fragment was then substituted into the vector pRRS*fokIR* to replace the DNA segment coding for the *Fok*I DNA-binding domain and, hence, form the *Ubx*-F_N hybrid gene (Fig. 17B). After transfection of competent RR1 cells with the ligation mix, several clones were identified by restriction analysis and their DNA sequences were confirmed by the dideoxy chain-termination method of Sanger et al. (Proc. Natl. Acad. Sci. USA 74:5463-67 (1977)). Second, the hybrid gene was amplified using the *Ubx*-F_N primers. The PCR-generated DNA was digested with *Nde*I/*Bam*HI and gel-purified. This fragment was then ligated into the *Nde*I/*Bam*HI-cleaved pET-15b vector. This construct will tag the hybrid protein with 6 consecutive histidine residues at the N-terminus. These serve as the affinity tag for purification of this protein by metal chelation chromatography using Novagen's His-bind™ resin. This His tag can be subsequently removed by thrombin. Competent BL21(DE3) cells were transformed with the ligation mix and several clones containing the recombinant DNA (Fig. 17B) were identified. These colonies were sick and grew

poorly in culture with a doubling time of about 45 minutes. After induction with 1 mM isopropyl-β-D-thiagalactoside (IPTG), the hybrid enzyme was purified to homogeneity using His-bind™ resin, phosphocellulose and gel-chromatography. The SDS/PAGE profile of the purified hybrid enzyme is shown in Fig. 18. The identity of the hybrid protein was further confirmed by probing the Western blot with rabbit antisera raised against *Fok*I endonuclease (data not shown).

Example XVI

Analysis of the DNA Sequence Preference of the *Ubx*-F_N Hybrid Enzyme

The linearized pUC13 derived substrates used to characterize *Ubx*-F_N are shown in Fig. 19. The derivatives were constructed by inserting a 30 bp DNA fragment containing a known *Ubx* recognition sequence 5'-TTAATGGTT-3' at the *Sma*I site of pUC13. Cleavage at the inserted *Ubx* site should yield ~1.8 kb and ~0.95 kb fragments as products. The agarose gel electrophoretic profile of the partial digests of the substrates by *Ubx*-F_N is shown in Fig. 19. In these reactions, the molar ratio of DNA was in large excess compared to the protein. The reaction condition was optimized to give a single double-stranded cleavage per substrate molecule. The linearized pUC13 DNA is cleaved into four fragments. The appearance of four distinct bands in the agarose gel electrophoretic profile indicates that *Ubx*-F_N binds DNA in a sequence-specific manner, and that there are two binding sites within the linearized pUC13 for the hybrid protein. This is further supported by the fact that the linearized pUC13 DNA substrate containing a single *Ubx* site is cleaved into six fragments. The two additional fragments (~1.8 kb and ~0.95 kb, respectively) could be

explained as resulting from the binding of the hybrid protein at the newly inserted *Ubx* site of pUC13 and cleaving near this site. As expected, the intensity of the bands increases with the number of 30 bp inserts in pUC13. The two putative *Ubx* binding sites in pUC13 and the inserted *Ubx* site are shown in Table 3 below. All these sites have 5'-TAAT-3' as their core sequence; and these preferred sites are consistent with those reported for the *Ubx* homeo domain. The affinity of *Ubx* homeo domain for these sites is modulated by the nucleotide bases surrounding the core site. It appears that the hybrid protein does turnover, since complete digestion is observed at longer time period or by increasing the protein concentration (data not shown). The cleavage is more specific at higher temperatures.

Example XVII

Analysis of the Cleavage Distance from the Recognition Site by the Hybrid Enzyme

To determine the distance of cleavage from the recognition site by *Ubx*-F_N, the cleavage products of the ^{32}P-labeled DNA substrates containing a single *Ubx* site were analyzed by PAGE (Fig. 20). The digestion products were analyzed alongside the Maxam-Gilbert's (G + A) sequencing reactions of the substrates. As expected, the cut sites are shifted away from the recognition site. On the 5'-TAAT-3' strand, *Ubx*-F_N cuts the DNA 3 nucleotides away from the recognition site while on the 5'-ATTA-3' strand it cuts 8, 9 or 10 nucleotide away from the recognition site. Analysis of th cut sites of *Ubx*-F_N based on the cleavage of the DNA substrate containing a single *Ubx* site is summarized in Fig. 20. The cleavage occurs 5' to the TAAT sequence and

is consistent with the way the *Ubx-F*$_{\underline{N}}$ hybrid protein was engineered (Fig. 16).

TABLE 1

Amino-terminal sequences of *Fok*I fragments from trypsin digestion

Fragment	Amino-terminal sequence	DNA substrate	SEQ ID NO
8 kDa	VSKIRTFG*VQNPGKFENLKRVVQVFDRS	-	16
58 kDa	SEAPCDAIIQ		17
25 kDa	QLVKSELEEK	+	18
41 kDa	VSKIRTFGWV		19
30 kDa	VSKIRTFGWV		19
11 kDa	FTRVPKRVY		20

TABLE 2

No. (1)	ENase-IIs[a] (isoschizomers) (2)	Protruding ends[c] (5)	Species (strain)[d] (6)	Co-produced ENases[c] (7)	Described MTases-II[F] [C or A] (8)	Commercial availability[G] (9)	References (10)
1.	AlwI (BinI) ((BthII)[i]	$5'N_1$	Acinetobacter lwoii			N,Z	Mo2, Ne3
2.	AlwXi (BbvI)	$5'N_4$	Acinetobacter lwofii X		(M.BbvI)		Mo6
3.	Alw261 (BsmAI)	$5'N_4$	Acinetobacter lwofii RFL26		M.Alw261 [C-5 and A-N6]		Gl1, Bi2
4.	BbsI (Bbvii)	$5'N_4$	Bacillus brevis (laterosporus NEB573)			N	Mo2, Ne3
5.	BbvI (AlwXI) (Uba11091), (Bsp4321)	$5'N4_4$	Bacillus brevis (ATTCC 9999)	BbvII	M.BbvI [C-5]	G,I,N,Z	Ba4, Do1, Do2, Gi2, Gi3, Ha4, Ha5, Ne3, Sc2, Va1
6.	BbvII (Bbv16I)[i] (BspVI)	$5'N_4$	Bacillus brevis 80	BbvI			Bu1, Bu2, Do2, Ma4
7.	BcefI	$5'N_1$	Bacillus cereus subsp. flourescens				Ve1, Ve2
8.	BccI		Bacteroides caccae			(N)	Mo2

No. (1)	ENase-IIs[a] (isoschizomers) (2)	Protruding ends[c] (5)	Species (strain)[d] (6)	Co-produced ENases[c] (7)	Described MTases-II[F] [C or A] (8)	Commercial availability[G] (9)	References (10)
9.	BcgI	$3'N_2$ $3'N_2$	Bacillus coagulants (NEB 566)			N	H. Kong, No3
10.	BinI (AlwI) (BthII)	$5'N_1$	bifidobacterium infantis			N	Bo2, Kh1, Kh2
11.	BsI (Eco31I)[j]	$5'N_4$	Bacillus stearothermophilus 6-55			N	H. Kong, Mo2, Ne3
12.	BsgI	$3'N_2$	Bacillus sphaericus GC			N	Sc2
13.	BsmAi (Alw26I)	$5'N_4$	Bacillus stearothermophilus A664 (NEB 481)			N	Ch1, Ko1, Ne3
14.	BspMI	$5'N_4$	Bacillus species M (NEB 356)	BspMII		N	Ha1, Ki2, Ki4, Ku1, Mc2, Mo2, Mo4, Mo7 Ne3
15.	EarI (Ksp632I)	$5'N_3$	Enterobacter aerogenes (NEB 450)			N	Ne3, Po3
16.	Eco31I (BsaI)[j]	$5'N_4$	Escherichia coli RFL31		M.Eco31I [C-5] and [A-N6]	F	Bi2, Bu3

No. (1)	ENase-IIs[a] (isoschizomers) (2)	Protruding ends[c] (5)	Species (strain)[d] (6)	Co-produced ENases[c] (7)	Described MTases-II[F] [C or A] (8)	Commercial availability[G] (9)	References (10)
17.	Eco57I (Bsp6II)[i] (Eco112I)[i] (Eco125I)[i] (FsfI)[i]	3'N_2	Escherichia coli RFL57,		M.Eco57I [A-N6]	F,N	Ja2, Ja3, Pe1, Pe2
18.	Esp3I	5'N_4	Erwinia sp RFL3		M.Esp3I [C-5, A-N6]	F,N	Bi2
19.	FaI	5'N_2	Flavobacterium aquatili				Bi2
20.	FokI (HinGuII)	5'N_4	Flavobacterium okeanokoites		M.FokI [A-N6]	A,M,N,S,U,Z	Ba4, Ha2, Ha3, Ka1, Ka2, Ki1, Ki3, Ki4, Ki5, Ki6, Ki7, Kr1, La1, Lo1, Lu1, Ma1, Ma3, Mc1, Ne3, Nw1, Po1, Po4, Po5, PO6, Sc3, sc4, sk1, su2, Su3, Su4, Sz1, Ve3, Ve4, Wi1

No. (1)	ENase-IIs[a] (isoschizomers) (2)	Protruding ends[c] (5)	Species (strain)[d] (6)	Co-produced ENases[c] (7)	Described MTases-II[f] [C or A] (8)	Commercial availability[g] (9)	References (10)
21.	GsuI (Bco35I)[i] (Bsp22I)[i] (Bsp28I)[i]	3'N_2	Gluconobacter dioxyacetonicus H015T		M.GsuI	F,N	Bi1, Ja1, Pe1, Pe2
22.	HgaI	5'N_5	Haemophilus gallinarum (ATCC14385)		M.HgaI (two MTases) [C-5]	N,Z	Ba4, Br1, Br6, Ko4, Kr1, Mo8, Ne1, Ne3, Su1, Ta1, To1, Ur1
23.	HinGuII (FokI)	5'N_4	Haemophilus infuenze GU				Na2
24.	HphI (NgoVII) (NgoBI)	3'N_1 (or blunt)	Haemophilus parahaemolyticus		M.HphI [A-N6]	N,Z	Ba2, Co1, Kl1, Ne2, Ne3, Ro1
25.	Ksp632I (EarI) BsrEI)[i]	5'N_3	Kluyvera sp.632			M	Bo1
26.	MboII (NcuI)[i] (TceI)[i]	3'N_1	Moraxella bovis (ATCC10900)	MboI	M.MboII [A-N6]	B,G,I,N, P, U,Z	Ba1, Br3, Br5, En1, Ga1, Ge1, Ha2, Mc1, Mc3, Na1, Na2, Ne2, Ne3, Sc1, Se1 Sm1
27.	MmeI	3'N_2	Methylophilus methyltrophus	MmeII		U	Bo3, Tu1

No. (1)	ENase-IIs[a] (isoschi-zomers) (2)	Protruding ends[c] (5)	Species (strain)[d] (6)	Co-produced ENases[c] (7)	Described MTases-II[F] [C or A] (8)	Commercial availability[G] (9)	References (10)
28.	MnlI	$3'N_1$	Moraxella nonliuefa-ciens (ATCC17953)			I,N,S,Z	Br2, Ne3, Sc2, Vi1, Ea1
29.	NgoVIII (HphI)	n.d.	Neisseria gonorrhoeae		M.NgoVIII		Ko2
30.	PleI	$5'N_1$	Pseudomonas	lemoignei (NEB418)		N	Mo6, Ne3
31.	RleAI	$3'N_3$	Rhizobium leguminosarum				Ve5
32.	SapI	$5'N_3$	Saccharo-polyspora sp.			N	Mo2, Ne3
33.	SfaNI (BscAI)[i]	$5'N_4$	Streptococcus faecalis ND547		M.SfaI	N,Z	Ba4, Ne3, Po5, Po6, Sc2, Sc3, Sc5, Sp1
34.	TaqII	$3'N_2$	Thermus aquaticus	TaqI		U	Ba2, My1
35.	Tth111II	$3'N_2$	Thermus thermophilus 111	Tth111I		Y,Z	Sh1, Sh2
36. Related ENases:[h]	Sts I	Streptococ-cus sanguis 54					

No. (1)	ENase-IIs[a] (isoschi-zomers) (2)	Protruding ends[c] (5)	Species (strain)[d] (6)	Co-produced ENases[c] (7)	Described MTases-II[f] [C or A] (8)	Commercial availability[g] (9)	References (10)
36.	BsmI (Asp35HI)[k]	3'N_1	Bacillus stearo-thermophilus NUB36			N	Gi1, Ha6, In1, MO7, My1, Ne3, Pa1
37.	BarI (BarSI)	3'N_1	Bacillus stearothermo-philus (NEB447)			N	Ne3, Po2

[a] Class-II restriction endonucleases (ENases-IIS) as listed (Ke1;Ro2). Isoschizomers are listed in parentheses (very recently discovered or incompletely characterized isoschizomers are in footnotes i-k). An ENase-IIS is defined as an enzyme which cuts at precise distance away from its recognition site, without cleaving this site. Enzymes in lines 36 and 37 ((Bsmi, Bsr, six Asp, and BscCI) do not fit this definition because one of the two cuts is within the recognition site, but they were included because several of their properties and applications are quite similar to those of enzymes 1-35. ENase in line 29 (NgoVII) was not described, but the M.Ngo VIII MTase appears to match the HphI). Genes coding for Eco57I and FokI were cloned (Ja3; Wi1). ENases EcqI, Eco57I and GsuI (and their isoschizomers?) require or are stimulated by AdoMet.

[b] The recognition sequences ae asymmetric [with exception of those marked S (in bp column) which display a partial symmetry (which might be incidental)], and are oriented so that the cut sites are to the right (downstream) of them. E.g., $\frac{GGATC(N)_4}{CCTAG(N)_5}$ (line 1), indicates that the cut on the upper strand is between 4th

TABLE 3

Ubx-binding Sites in pUC13

Sequence	Remarks
5'-TTAATGTCA-3'	putative *Ubx* sites present in pUC13
5'-TTAATGAAT-3'	
5'-TTAATGGTT-3'	*Ubx* site inserted at the *Sma*I site of pUC13

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Chandrasegaran, Srinivasan

(ii) TITLE OF INVENTION: Functional Domains in FokI Restriction Endonuclease

(iii) NUMBER OF SEQUENCES: 48

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Cushman, Darby & Cushman
(B) STREET: 1100 New York Ave., N.W.
(C) CITY: Washington
(D) STATE: D.C.
(E) COUNTRY: USA
(F) ZIP: 20005-3918

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/126,564
(B) FILING DATE: 27-SEPTEMBER-93
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kokulis, Paul N.
(B) REGISTRATION NUMBER: 16,773
(C) REFERENCE/DOCKET NUMBER: PNK/4130/82506/CLB

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 202-861-3503
(B) TELEFAX: 202-822-0944
(C) TELEX: 6714627 CUSH

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGATG 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCTAC 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 18..35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
CCATGGAGGT TTAAAAT ATG AGA TTT ATT GGC AGC     35
                   Met Arg Phe Ile Gly Ser
                    1               5
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Arg Phe Ile Gly Ser 6
1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATACCATGGG AATTAAATGA CACAGCATCA 30

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 22..42

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
TAGGATCCGG AGGTTTAAAA T ATG GTT TCT AAA ATA AGA ACT        42
                        Met Val Ser Lys Ile Arg Thr
                         1               5
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Val Ser Lys Ile Arg Thr 7
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TAGGATCCTC ATTAAAAGTT TATCTCGCCG TTATT 35

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Asn Asn Gly Glu Ile Asn Phe 7
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCTCTGGATG CTCTC 15

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GAGAGCATCC AGAGG 15

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TAATTGATTC TTAA 14

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ATTAAGAATC AATT 14

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CCTCTGGATG CTCTCAAAAA AAAAAAAAAA 30

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GAGAGCATCC AGAGGAAAAA AAAAAAAAAA 30

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Val Ser Lys Ile Arg Thr Phe Gly Xaa Val Gln Asn Pro Gly Lys
1 5 10 15

Phe Glu Asn Leu Lys Arg Val Val Gln Val Phe Asp Arg Ser
20 25

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Ser Glu Ala Pro Cys Asp Ala Ile Ile Gln 10

1 5 10

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Gln Leu Val Lys Ser Glu Leu Glu Glu Lys 10
1 5 10

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Val Ser Lys Ile Arg Thr Phe Gly Trp Val 10
1 5 10

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Phe Thr Arg Val Pro Lys Arg Val Tyr 9
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Glu Glu Lys 3
1

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Lys Ser Glu Leu 4
1

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Lys Ser Glu Leu Glu Glu Lys 7
1 5

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TAGCAACTAA TTCTTTTTGG ATCTT 25

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CCATCGATAT AGCCTTTTTT ATT 23

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GCTCTAGAGG ATCCGGAGGT 20

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CGCAGTGTTA TCACTCAT 18

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CTTGGTTGAG TACTCACC 18

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

ACCGAGCTCG AATTCACT 18

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GATTTCGGCC TATTGGTT 18

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 579 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Met Val Ser Lys Ile Arg Thr Phe Gly Trp Val Gln Asn Pro Gly
1 5 10 15

Lys Phe Glu Asn Leu Lys Arg Val Val Gln Val Phe Asp Arg Asn
20 25 30
Ser Lys Val His Asn Glu Val Lys Asn Ile Lys Ile Pro Thr Leu
35 40 45
Val Lys Glu Ser Lys Ile Gln Lys Glu Leu Val Ala Ile Met Asn
50 55 60
Gln His Asp Leu Ile Tyr Thr Tyr Lys Glu Leu Val Gly Thr Gly
65 70 75
Thr Ser Ile Arg Ser Glu Ala Pro Cys Asp Ala Ile Ile Gln Ala
80 85 90
Thr Ile Ala Asp Gln Gly Asn Lys Lys Gly Tyr Ile Asp Asn Trp
95 100 105
Ser Ser Asp Gly Phe Leu Arg Trp Ala His Ala Leu Gly Phe Ile
110 115 120
Glu Tyr Ile Asn Lys Ser Asp Ser Phe Val Ile Thr Asp Val Gly
125 130 135
Leu Ala Tyr Ser Lys Ser Ala Asp Gly Ser Ala Ile Glu Lys Glu

140 145 150
Ile Leu Ile Glu Ala Ile Ser Ser Tyr Pro Pro Ala Ile Arg Ile
155 160 165
Leu Thr Leu Leu Glu Asp Gly Gln His Leu Thr Lys Phe Asp Leu
170 175 180
Gly Lys Asn Leu Gly Phe Ser Gly Glu Ser Gly Phe Thr Ser Leu
185 190 195
Pro Glu Gly Ile Leu Leu Asp Thr Leu Ala Asn Ala Met Pro Lys
200 205 210
Asp Lys Gly Glu Ile Arg Asn Asn Trp Glu Gly Ser Ser Asp Lys
215 220 225
Tyr Ala Arg Met Ile Gly Gly Trp Leu Asp Lys Leu Gly Leu Val
230 235 240
Lys Gln Gly Lys Lys Glu Phe Ile Ile Pro Thr Leu Gly Lys Pro
245 250 255
Asp Asn Lys Glu Phe Ile Ser His Ala Phe Lys Ile Thr Gly Glu
260 265 270
Gly Leu Lys Val Leu Arg Arg Ala Lys Gly Ser Thr Lys Phe Thr
275 280 285
Arg Val Pro Lys Arg Val Tyr Trp Glu Met Leu Ala Thr Asn Leu
290 295 300
Thr Asp Lys Glu Tyr Val Arg Thr Arg Arg Ala Leu Ile Leu Glu
305 310 315
Ile Leu Ile Lys Ala Gly Ser Leu Lys Ile Glu Gln Ile Gln Asp
320 325 330
Asn Leu Lys Lys Leu Gly Phe Asp Glu Val Ile Glu Thr Ile Glu
335 340 345
Asn Asp Ile Lys Gly Leu Ile Asn Thr Gly Ile Phe Ile Glu Ile
350 355 360
Lys Gly Arg Phe Tyr Gln Leu Lys Asp His Ile Leu Gln Phe Val
365 370 375
Ile Pro Asn Arg Gly Val Thr Lys Gln Leu Val Lys Ser Glu Leu
380 385 390
Glu Glu Lys Lys Ser Glu Leu Arg His Lys Leu Lys Tyr Val Pro
395 400 405
His Glu Tyr Ile Glu Leu Ile Glu Ile Ala Arg Asn Ser Thr Gln
410 415 420
Asp Arg Ile Leu Glu Met Lys Val Met Glu Phe Phe Met Lys Val
425 430 435
Tyr Gly Tyr Arg Gly Lys His Leu Gly Gly Ser Arg Lys Pro Asp
440 445 450
Gly Ala Ile Tyr Thr Val Gly Ser Pro Ile Asp Tyr Gly Val Ile
455 460 465
Val Asp Thr Lys Ala Tyr Ser Gly Gly Tyr Asn Leu Pro Ile Gly
470 475 480
Gln Ala Asp Glu Met Gln Arg Tyr Val Glu Glu Asn Gln Thr Arg
485 490 495
Asn Lys His Ile Asn Pro Asn Glu Trp Trp Lys Val Tyr Pro Ser
500 505 510
Ser Val Thr Glu Phe Lys Phe Leu Phe Val Ser Gly His Phe Lys
515 520 525
Gly Asn Tyr Lys Ala Gln Leu Thr Arg Leu Asn His Ile Thr Asn
530 535 540
Cys Asn Gly Ala Val Leu Ser Val Glu Glu Leu Leu Ile Gly Gly
545 550 555
Glu Met Ile Lys Ala Gly Thr Leu Thr Leu Glu Glu Val Arg Arg

560 565 570
Lys Phe Asn Asn Gly Glu Ile Asn Phe
575

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Lys Gln Leu Val Lys Ser Glu Leu Glu Glu Lys 11
1 5 10

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

AAGCAACTAG TCAAAAGTGA ACTGGAGGAG AAG 33

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Leu Val Lys Ser Glu Leu Lys Ser Glu Leu Glu Glu Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GGACTAGTCA AATCTGAACT TAAAAGTGAA CTGGAGGAGA AG 42

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Leu Val Lys Ser Glu Leu Glu Glu Lys Lys Ser Glu Leu Glu
1 5 10

Glu Lys
15

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GGACTAGTCA AATCTGAACT TGAGGAGAAG AAAAGTGAAC TGGAGGAGAA G 51

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Asn Phe Xaa Xaa 4
1

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

TTGAAAATTA CTCCTAGGGG CCCCCCT 27

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GGATGNNNNNNNNNNNNNNNNNN 23

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

TACCTGCAGC GGAGGTTTAA AAT ATG CGA AGA CGC GGC CGA 41
Met Arg Arg Arg Gly Arg
1 5

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

```
T TAC TTC GAC TTC TTC CTC TAG GTT GAT CAG AT    33
  Met Lys Leu Lys Lys Glu Ile Gln Leu Val
   1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

```
CCA CGG CAT ATG CGA AGA CGC GGC CGA              27
            Met Arg Arg Arg Gly Arg
             1               5
```

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

```
TTA TTG CCG CTC TAT TTG AAA ATT ACT CCTAGG AT  35
Asn Asn Gly Glu Ile Asn Phe
 1               5
```

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

AGAGGAGGTA ATGGG 15

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

ATTAAGGGGG GAAGAG 16

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CTCTAGAGGA TCCCCGCGCT TAATGGTTTT TGC 33

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GAGATCTCCT AGGGGCGCGA ATTACCAAAA ACG 33

* * * * *

All publications mentioned hereinabove are hereby incorporated by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art that various changes in form and detail can be made without departing from the true scope of the invention.

WHAT IS CLAIMED IS:

1. An isolated DNA segment encoding the recognition domain of a Type IIS endonuclease which contains the sequence-specific recognition activity of said Type IIS endonuclease.

2. The DNA segment of claim 1 wherein said Type IIS endonuclease is *Fok*I restriction endonuclease.

3. The DNA segment of claim 2 wherein the encoded protein has a molecular weight of about 41 kilodaltons as determined by SDS polyacrylamide gel electrophoresis.

4. The DNA segment of claim 3 which encodes amino acids 1-382 of the *Fok*I restriction endonuclease.

5. An isolated DNA segment encoding the catalytic domain of a Type IIS endonuclease which contains the cleavage activity of said Type IIS endonuclease.

6. The DNA segment of claim 5 wherein said Type IIS endonuclease is *Fok*I restriction endonuclease.

7. The DNA segment of claim 6 wherein the encoded protein has a molecular weight of about 25 kilodaltons as determined by SDS-polyacrylamide gel electrophoresis.

8. The DNA segment of claim 7 which encodes amino acids 383-578 of the *Fok*I restriction endonuclease.

9. An isolated protein consisting essentially of the N-terminus of the *Fok*I restriction endonuclease which protein has the sequence-specific recognition activity of said endonuclease.

10. The protein of claim 9 which is amino acids 1-382 of the *Fok*I restriction endonuclease.

11. An isolated protein consisting essentially of the C-terminus of the *Fok*I restriction endonuclease which protein has the cleavage activity of said endonuclease.

12. The protein of claim 11 which is amino acids 383-578 of the *Fok*I restriction endonuclease.

13. A DNA construct comprising:

(i) a first DNA segment encoding the catalytic domain of a Type IIS endonuclease which contains the cleavage activity of said Type IIS endonuclease;

(ii) a second DNA segment encoding a sequence-specific recognition domain other than the recognition domain of said Type IIS endonuclease; and

(iii) a vector

wherein said first DNA segment and said second DNA segment are operably linked to said vector so that a single protein is produced.

14. The DNA construct according to claim 13 wherein said Type IIS endonuclease is *Fok*I restriction endonuclease..

15. The DNA construct according to claim 14 wherein said recognition domain is selected from the group consisting of: zinc finger motifs, homeo domain motifs, DNA binding domains of repressors, POU domain motifs (eukaryotic transcription regulators), DNA binding domains of oncogenes and naturally occurring sequence-specific DNA binding proteins that recognize >6 base pairs.

16. The DNA construct according to claim 15 wherein said recognition domain is the homeo domain of *Ubx*.

17. A procaryotic cell comprising:

(i) a first DNA segment encoding the catalytic domain of a Type IIS endonuclease which contains the cleavage activity of said Type IIS endonuclease;

(ii) a second DNA segment encoding a sequence-specific recognition domain other than the recognition domain of said Type IIS endonuclease; and

(iii) a vector

wherein said first DNA segment and said second DNA segment are operably linked to said vector so that a single protein is produced.

18. The procaryotic cell of claim 17 wherein said first DNA segment encodes the catalytic domain (F_N) of *Fok*I, and said second DNA segment encodes the homeo domain of *Ubx*.

19. A hybrid restriction enzyme comprising the catalytic domain of a Type IIS endonuclease which contains the cleavage activity of said Type IIS endonuclease covalently linked to a

recognition domain of an enzyme other than said Type IIS endonuclease.

20. The hybrid restriction enzyme of claim 19 wherein said recognition domain, which comprises part of said hybrid restriction enzyme, is selected from the group consisting of: zinc finger motifs, homeo domain motifs, POU domain motifs, DNA binding domains of repressors, DNA binding domains of oncogenes and naturally occurring sequence-specific DNA binding proteins that recognize >6 base pairs.

21. The hybrid restriction enzyme of claim 20 wherein said recognition domain is the homeo domain of *Ubx*.

22. The hybrid restriction enzyme of claim 21 wherein said Type II endonuclease is *Fok*I restriction endonuclease and said hybrid enzyme is *Ubx*-F_N.

23. A DNA construct comprising:

(i) a first DNA segment encoding the catalytic domain of a Type IIS endonuclease which contains the cleavage activity of said Type IIS endonuclease;

(ii) a second DNA segment encoding a sequence-specific recognition domain other than the recognition domain of said Type IIS endonuclease;

(iii) a third DNA segment comprising one or more codons, wherein said third DNA segment is inserted between said first DNA segment and said second DNA segment; and

(iv) a vector

wherein said first DNA segment, said second DNA segment and said third DNA segment are

operably linked to said vector so that a single protein is produced.

24. The DNA construct according to claim 23 wherein said Type IIS endonuclease is *Fok*I restriction endonuclease.

25. The DNA construct according to claim 24 wherein said third DNA segment consists essentially of four codons.

26. The DNA construct according to claim 25 wherein said four codons of said third DNA segment are inserted at nucleotide 1152 of the gene encoding said endonuclease.

27. The DNA construct according to claim 24 wherein said third DNA segment consists essentially of 7 codons.

28. The DNA construct according to claim 27 wherein said 7 codons of said third DNA segment are inserted at nucleotide 1152 of the gene encoding said endonuclease.

29. The DNA construct according to claim 24 wherein said recognition domain is selected from the group consisting of: zinc finger motifs, homeo domain motifs, POU domain motifs, DNA binding domains of repressors, DNA binding domains of oncogenes and naturally occurring sequence-specific DNA binding proteins that recognize >6 base pairs.

30. A procaryotic cell comprising:

(i) a first DNA segment encoding the catalytic domain of a Type IIS endonuclease which

contains the cleavage activity of said Type IIS endonuclease;

(ii) a second DNA segment encoding a sequence-specific recognition domain other than the recognition domain of said Type IIS endonuclease;

(iii) a third DNA segment comprising one or more codons, wherein said third DNA segment is inserted between said first DNA segment and said second DNA segment; and

(iv) a vector

wherein said first DNA segment, said second DNA segment, and said third DNA segment are operably linked to said vector so that a single protein is produced.

31. The procaryotic cell of claim 30 wherein said third DNA segment consists essentially of four codons.

32. The procaryotic cell of claim 30 wherein said third DNA segment consists essentially of seven codons.

33. An isolated hybrid Type IIS endonuclease produced by the procaryotic cell of claim 30.

34. An isolated DNA segment encoding the N-terminus of a Type IIS endonuclease which contains the sequence-specific recognition activity of said Type II endonuclease, said Type II endonuclease being *Fok*I restriction endonuclease and having a molecular weight of about 41 kilodaltons as measured by SDS-polyacrylamide gel electrophoresis.

35. An isolated DNA segment encoding the C-terminus of a Type IIS endonuclease which contains

the cleavage activity of said Type IIS endonuclease, said Type II endonuclease being *Fok*I restriction endonuclease and having a molecular weight of about 25 kilodaltons as determined by SDS-polyacrylamide gel electrophoresis.

36. An isolated protein consisting essentially of the N-terminus of the *Fok*I restriction endonuclease which protein has the sequence-specific recognition activity of said endonuclease and which protein is amino acids 1-382 of said *Fok*I restriction endonuclease.

37. An isolated protein consisting essentially of the C-terminus of the *Fok*I restriction endonuclease which protein has the nuclease activity of said endonuclease and which protein is amino acids 383-578 of said *Fok*I restriction endonuclease.

FIG. 1

FokIM

5' primer

```
          NcoI         7-bp spacer
5' TA  CCATGG AGGT  TTAAAAT  ATG  AGA  TTT  ATT  GGC  AGC
          RBS                Met  Arg  Phe  Ile  Gly  Ser
```

3' primer

```
        18-bp complement           NcoI
3' ACT ACG ACA CAG TAA ATT AAG  GGTACC  ATA  5'
```

FokIR

5' primer

```
     BamHI     RBS      7-bp spacer
5' TA GGATCC  GGAGGT  TTAAAAT  ATG  GTT  TCT  AAA  ATA  AGA  ACT
                               Met  Val  Ser  Lys  Ile  Arg  Thr
```

3' primer

```
            Complementary Strand                       BamHI
3' TTA  TTG  CCG  CTC  TAT  TTG  AAA  ATT  ACT   CCTAGG  AT  5'
   Asn  Asn  Gly  Glu  Ile  Asn  Phe
```

FIG. 2A

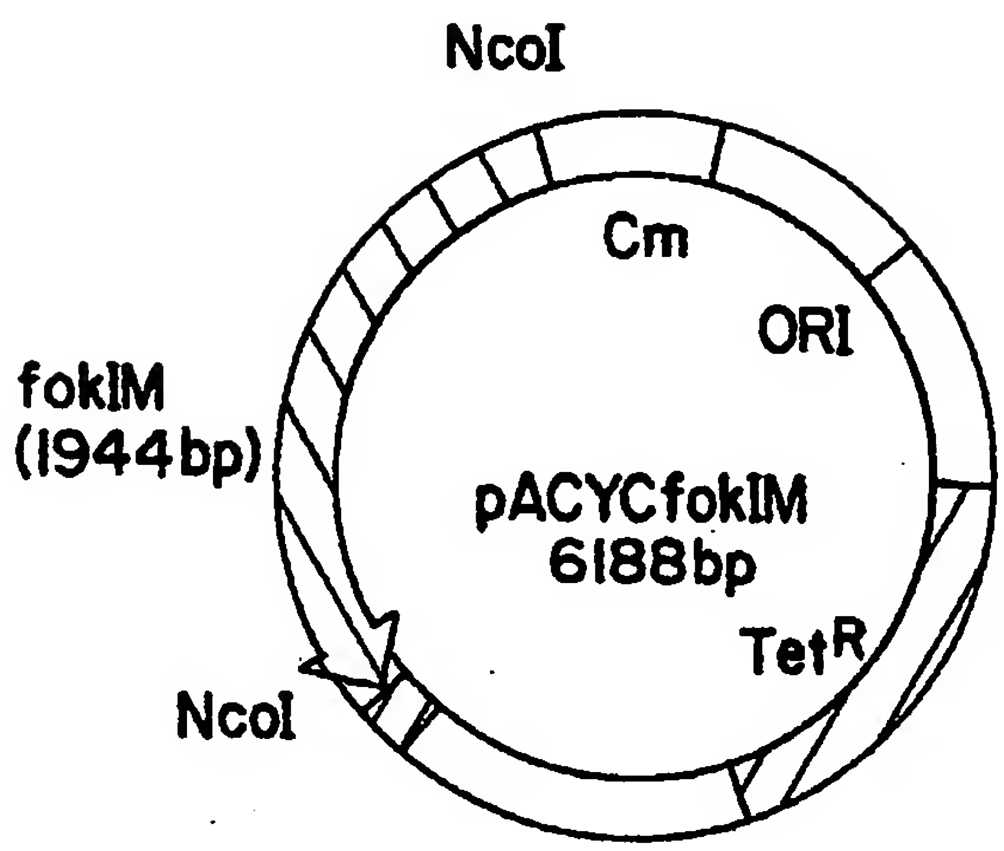


FIG. 2B

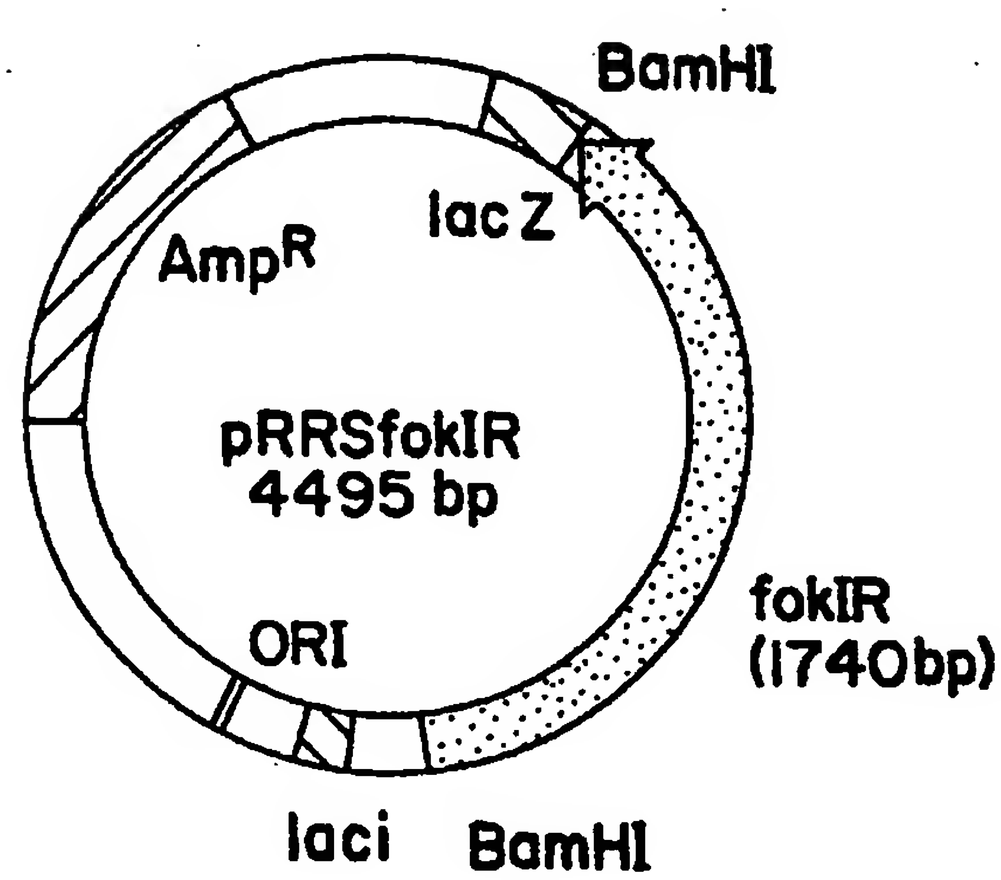


FIG. 2C

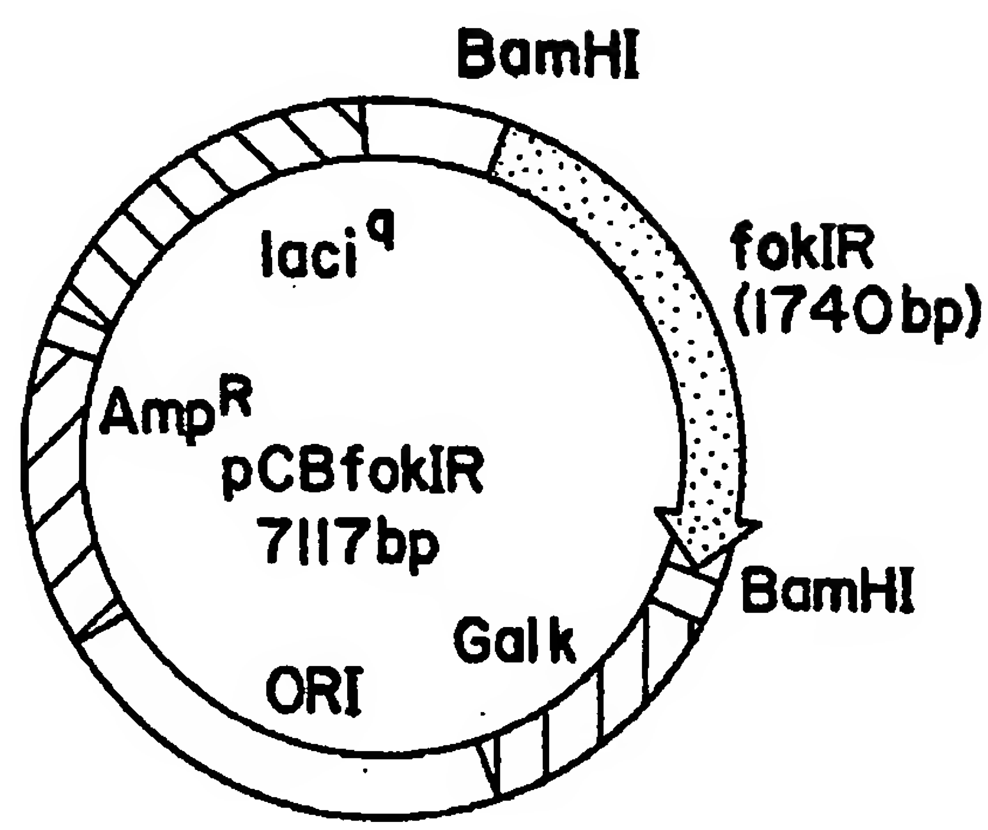


FIG. 3

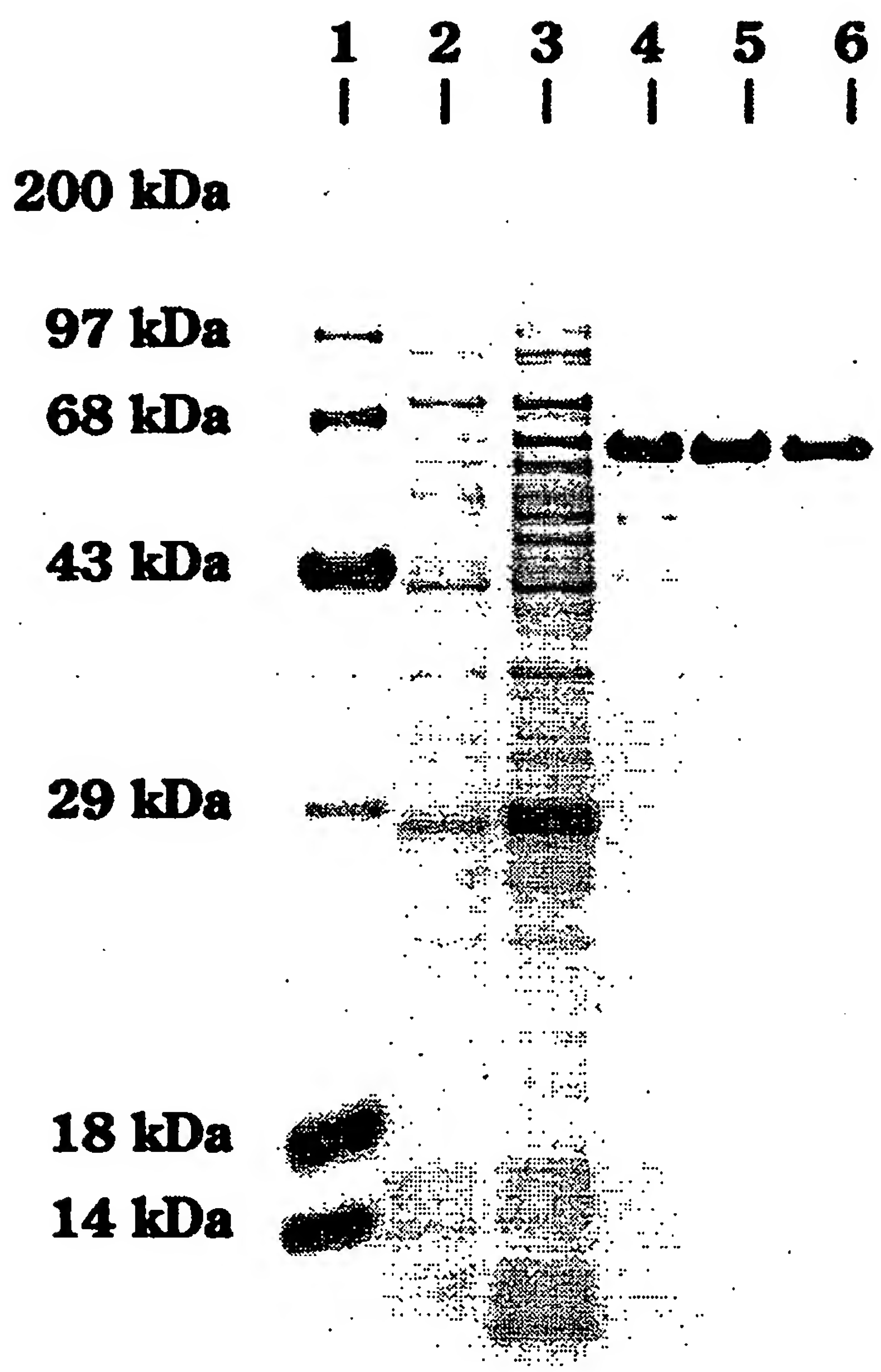


FIG. 4

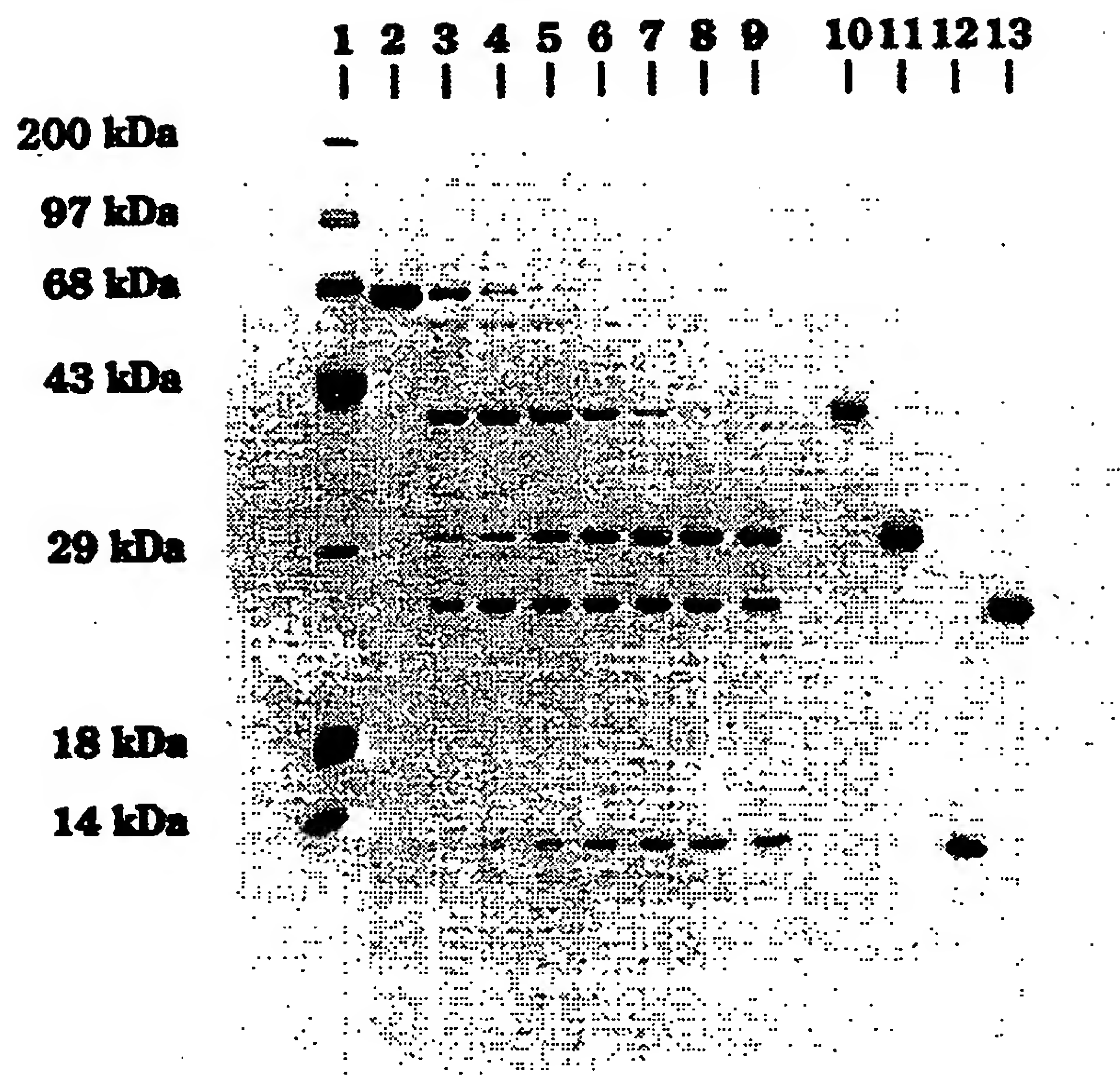

1 2 3 4 5 6 7 8 9 10 11 12 13
200 kDa
97 kDa
68 kDa
43 kDa
29 kDa
18 kDa
14 kDa

FIG. 5

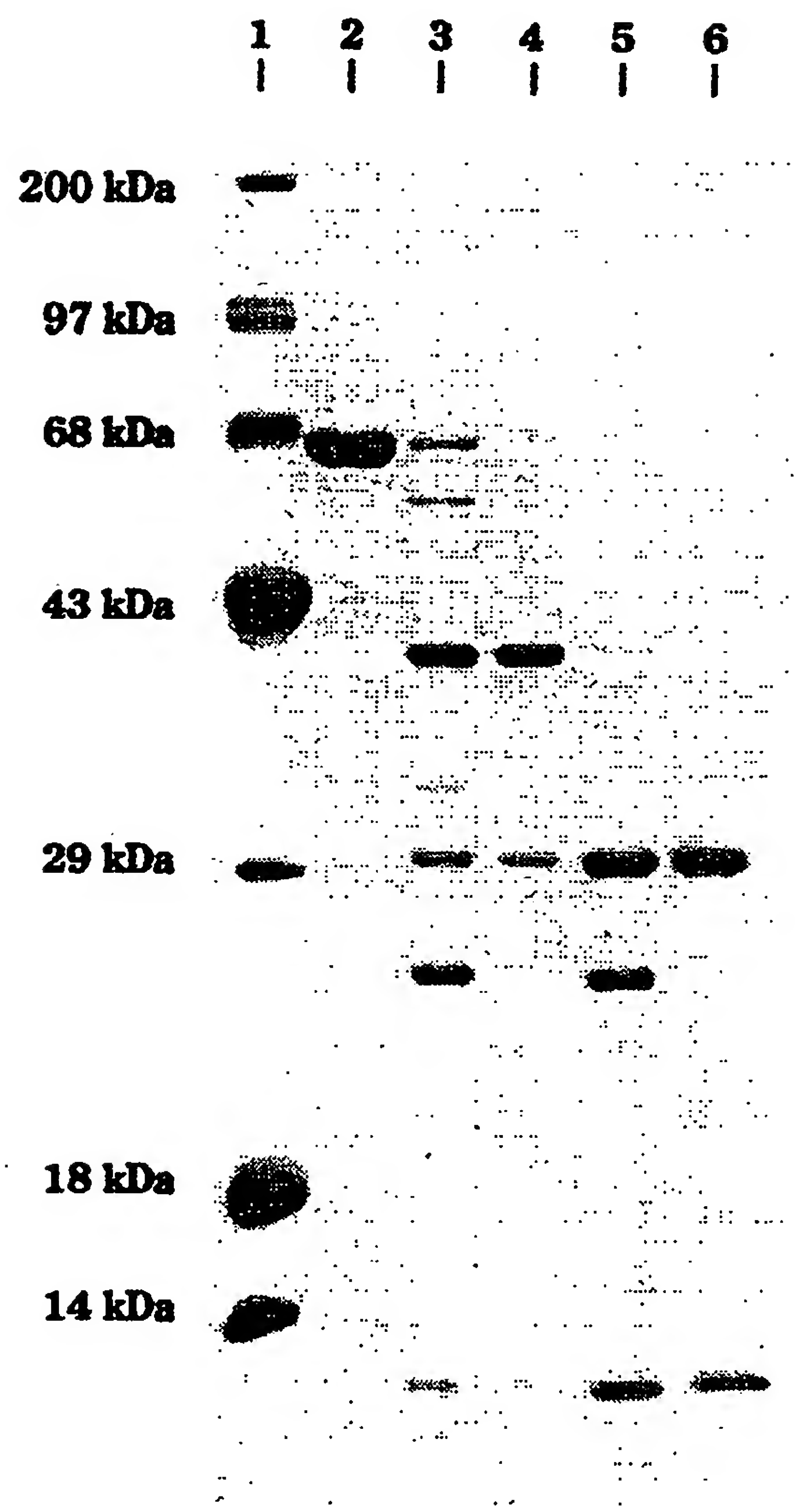


FIG.6A

-$MgCl_2$ +$MgCl_2$

1 2 3 4 5 6 7 8 9

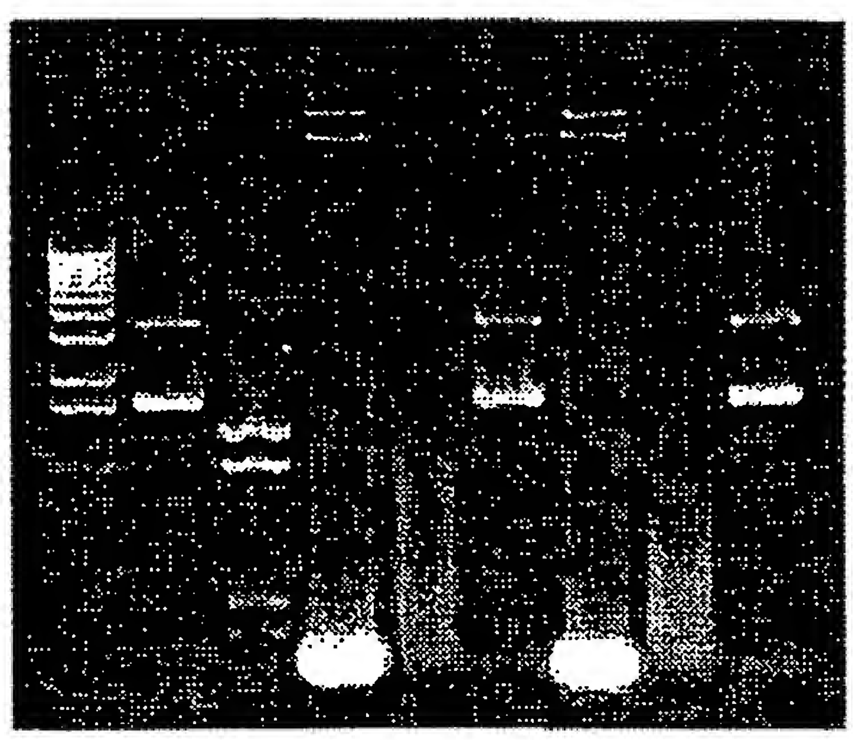


FIG.6B

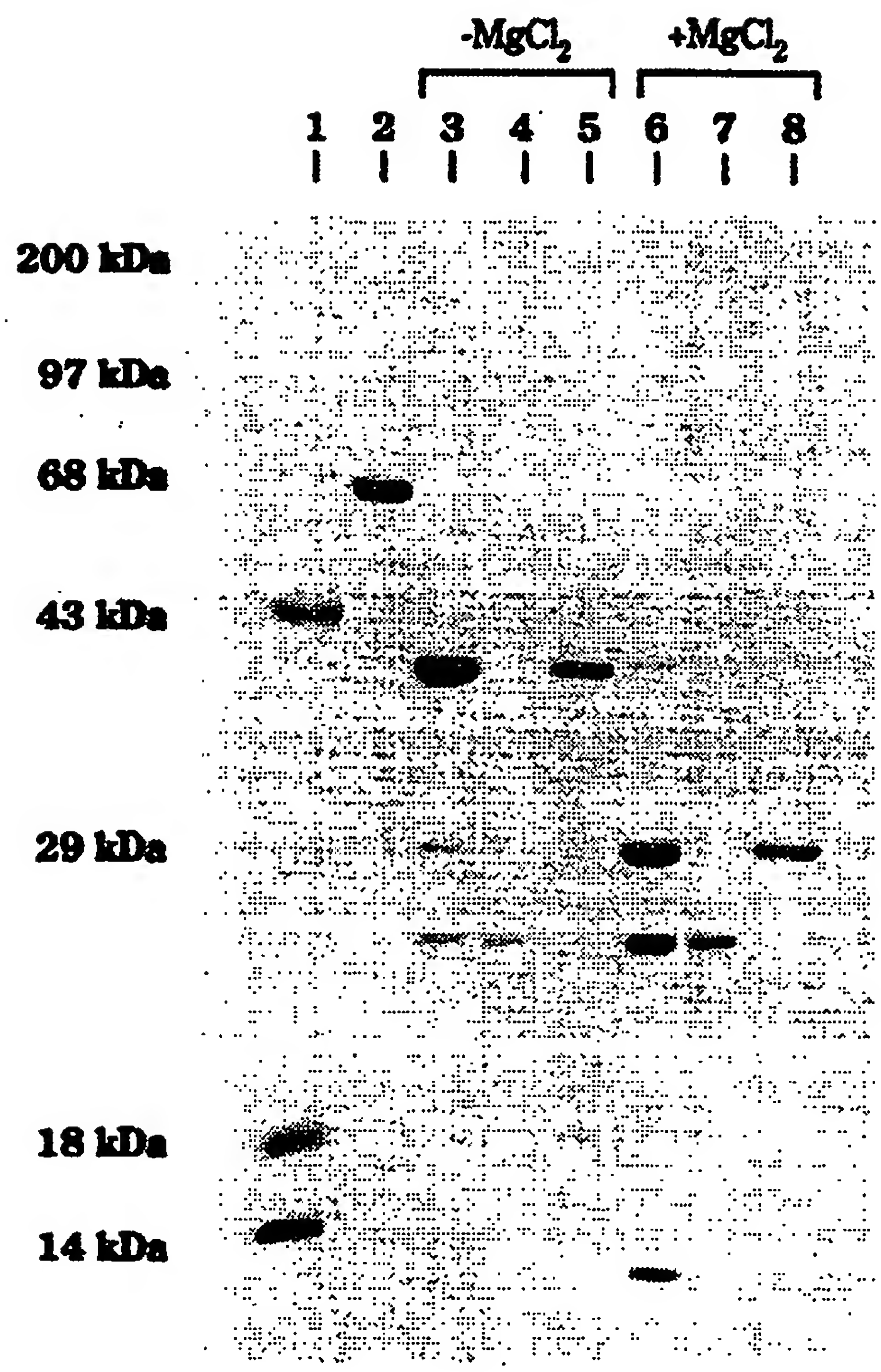

-MgCl$_2$
+MgCl$_2$
1 2 3 4 5 6 7 8
200 kDa
97 kDa
68 kDa
43 kDa
29 kDa
18 kDa
14 kDa

FIG.7A FIG.7B

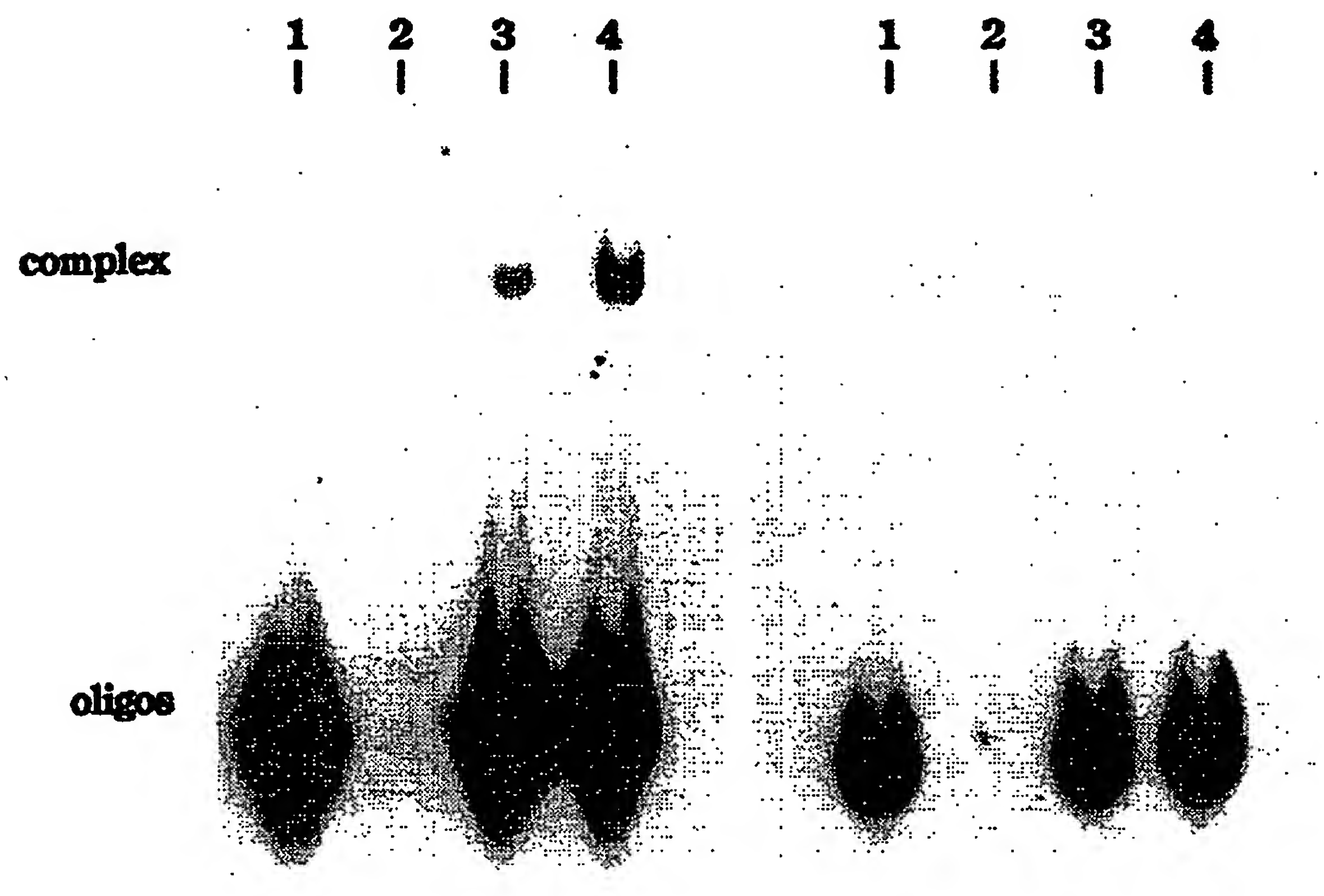


FIG. 8

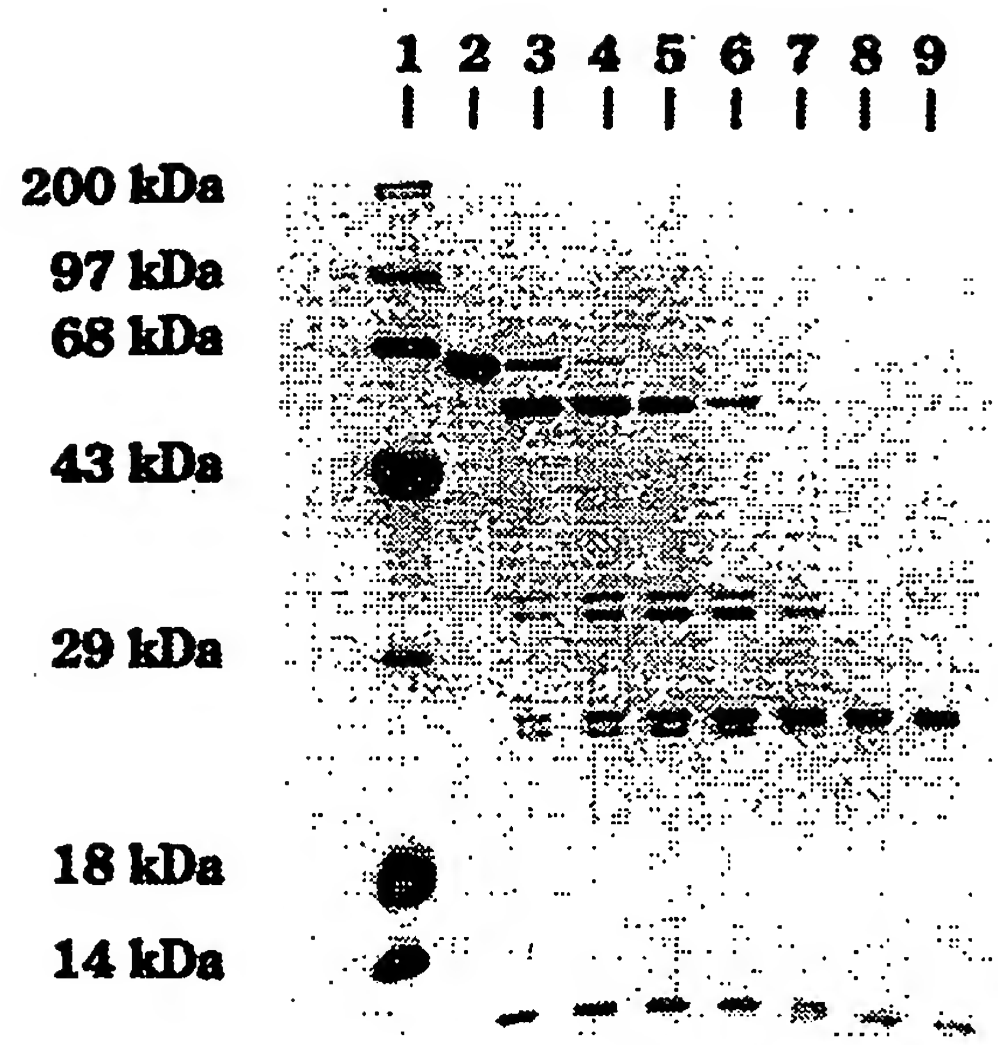
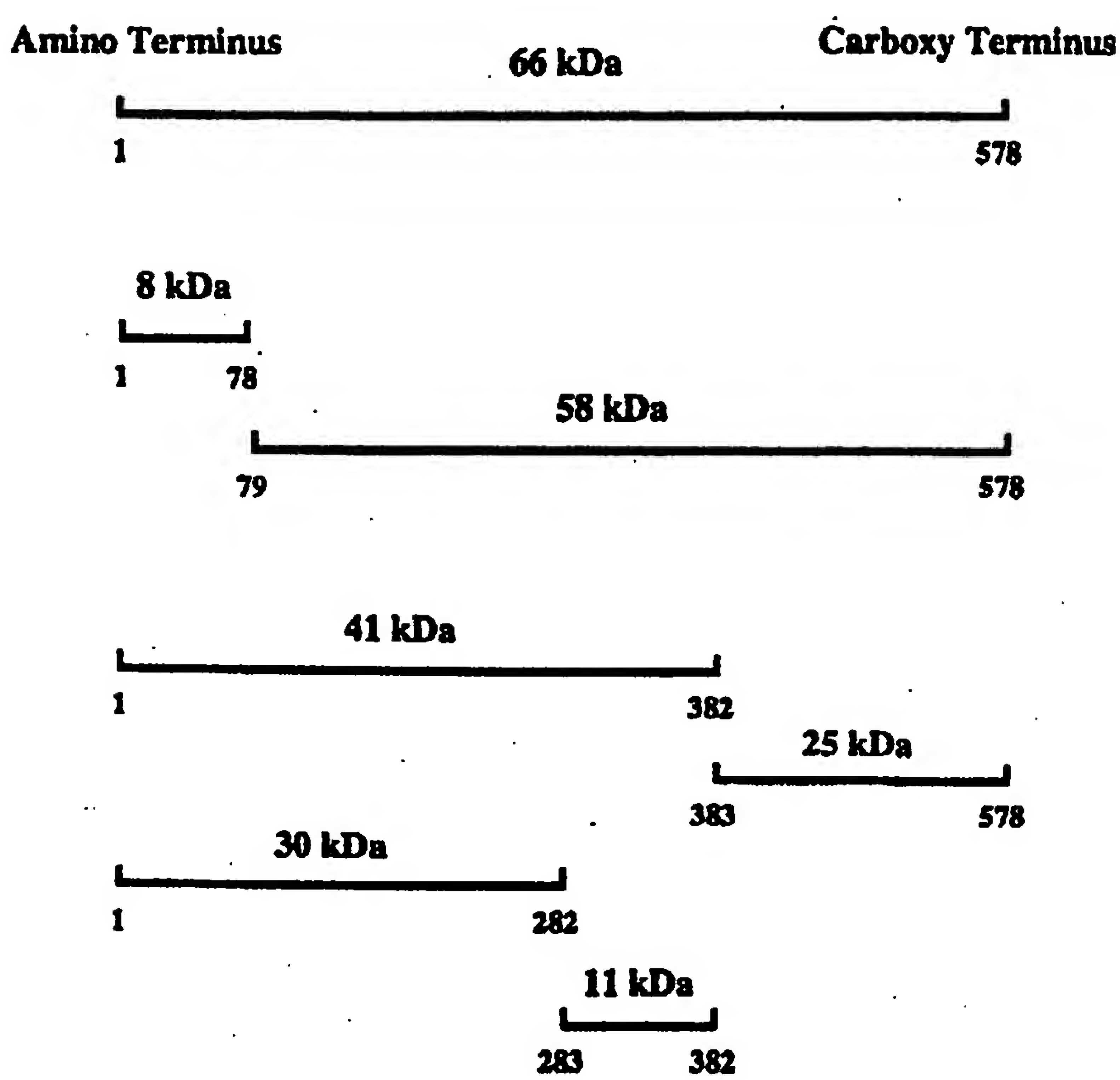


FIG. 9

*Fok*I endonuclease



```
MVSKIRTFGWVQNPGKFENLKRVVQVFDRNSKVHNEVKNIKIPTLVKESKIQKELVAIMNQHDLIYTYKELVGTGTSIR
ss.ss.ssss......hhhhhhhhhhh......sssssss...hhhhhhhhhhhhhhhhh..sssssssssssssss

SEAPCDAIIQATIADQGNKKGYIDNWSSDGFLRWAHALGFIEYINKSDSFVITDVGLAYSKSADGSAIEKEILIEAISS
.....hhhhhhhhh...................hhhhhhhhhhhh....sssssssss......hhhhhhhhhhhhhh.

YPPAIRILTLLEDGQHLTKFDLGKNLGFSGESGFTSLPEGILLDTLANAMPKDKGEIRNNWEGSSDKYARMIGGWLDKL
....sssssshhhh...................sss....hhhhhhhhhhh........hhhh.....hhhhh...hhhh

GLVKQGKKEFIIPTLGKPDNKEFISHAFKITGEGLKVLRRAKGSTKFTRVPKRVYWEMLATNLTDKEYVRTRRALILEI
hhhhhh...sss..........hhhhsssss...hhhhhhhh..........hhhhhhhhhh..hhhhssshhhhhhh

LIKAGSLKIEQIQDNLKKLGFDEVIETIENDIKGLINTGIFIEIKGRFYQLKDHILQFVIPNRGVTKQLVKSELEEKKS
hhhh..hhhhhhhhhhhhhhhhhhhhhhhhhh...ssssssssssssssssssssss......hhhhhhhhhhhh

ELRHKLKYVPHEYIELIEIARNSTQDRILEMKVMEFFMKVYGYRGKHLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGG
hhhhhh.....hhhhhhhhhh.hhhhhhhhhhhhhhhhhhhh..............ssss.....sssss.......

YNLPIGQADEMQRYVEENQTRNKHINPNEWWKVYPSSVTEFKFLFVSGHFKGNYKAQLTRLNHITNCNGAVLSVEELLI
........hhhhhhhhhhh.........hhhsss....sssssss.......hhhhhhhhhhhhh...hhhhhhhh

GGEMIKAGTLTLEEVRRKFNNGEINF
hhhhhh.hhhhhhhhhh.........
```

FIG. 10

fokIR nt sequence

```
        K   Q   L   V   K   S   E   L   E   E   K
5'-......AAG CAA CTA GTC AAA AGT GAA CTG GAG GAG AAG...... -3'
                 SpeI
```

5' primers:

oligonucleotide for 4-codon insertion

```
        L   V   K   S   E   L   K   S   E   L   E   E   K
5'- GGA CTA GTC AAA TCT GAA CTT AAA AGT GAA CTG GAG GAG AAG -3'
        SpeI                    21-bp complement
```

oligonucleotide for 7-codon insertion

```
        L   V   K   S   E   L   E   E   K   K   S   E   L   E   E   K
5'- GGA CTA GTC AAA TCT GAA CTT GAG GAG AAG AAA AGT GAA CTG GAG GAG AAG -3'
        SpeI                                21-bp complement
```

3' primer:

```
    N   F   Ter Ter BamHI
3'- TTG AAA ATT ACT CCTAGGGGCCCCCT -5'
                        XmaI
```

FIG. 11

FIG. 12

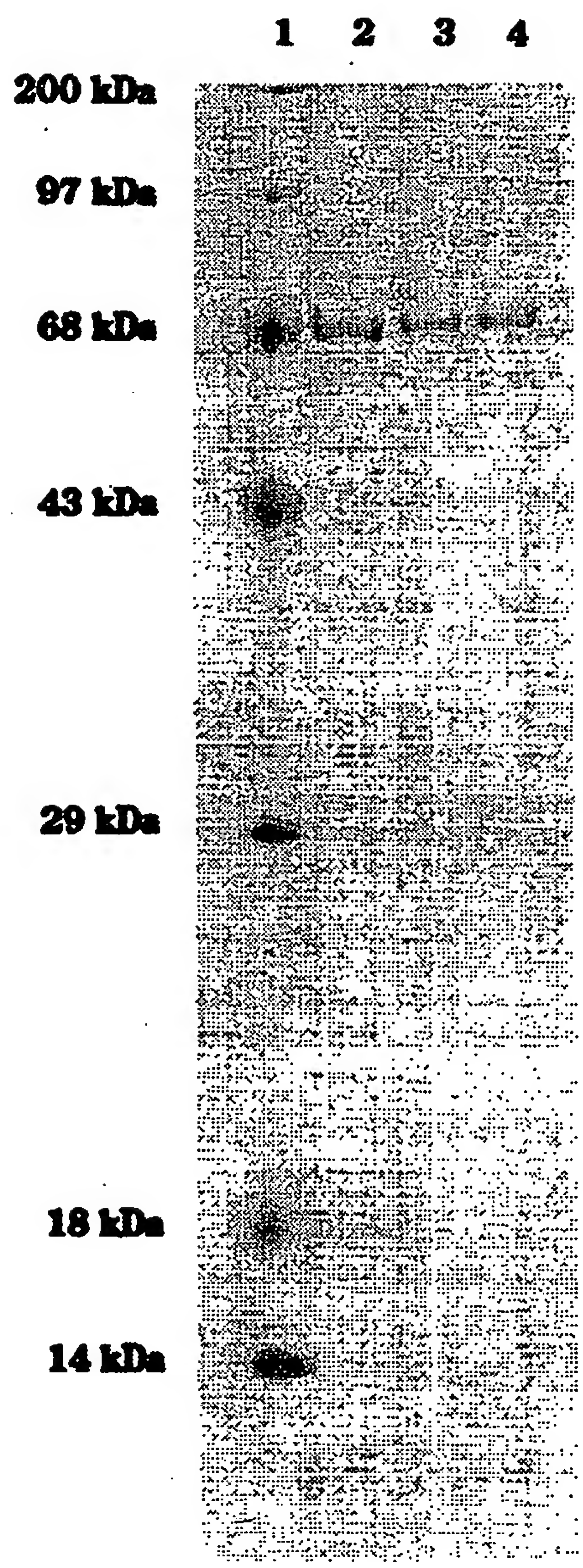


FIG. 13A

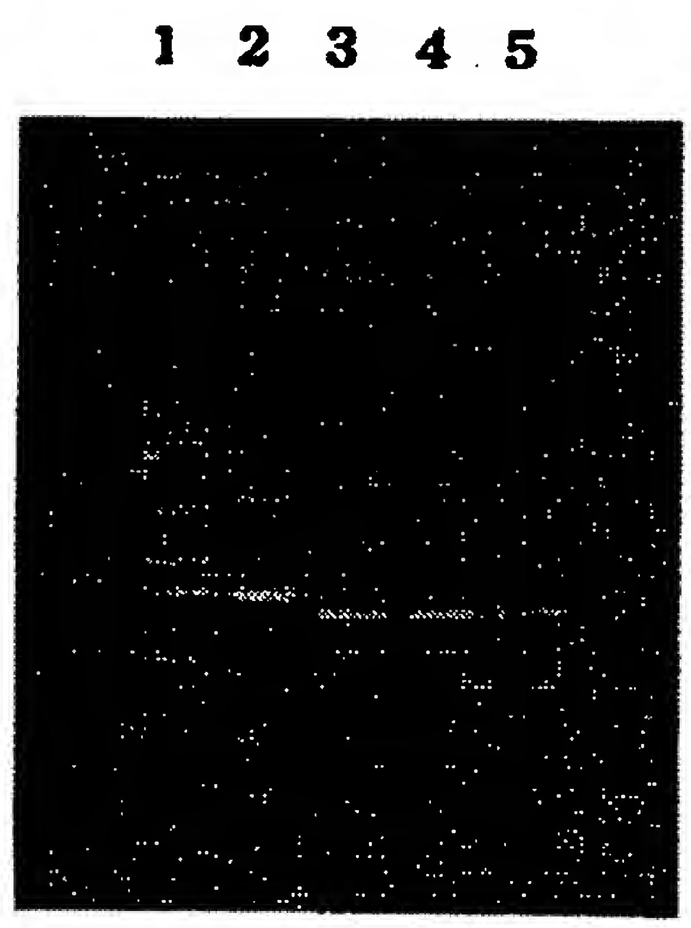


FIG. 13B

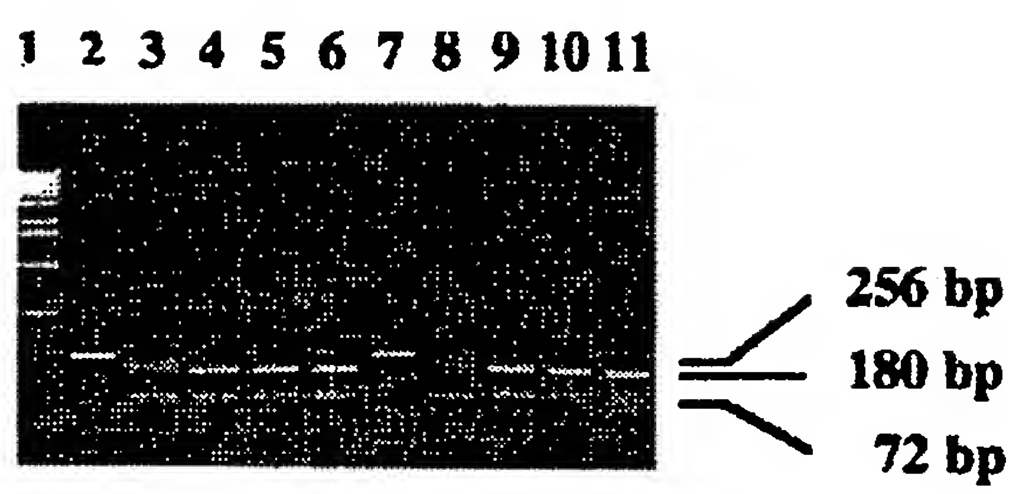


FIG. 13C

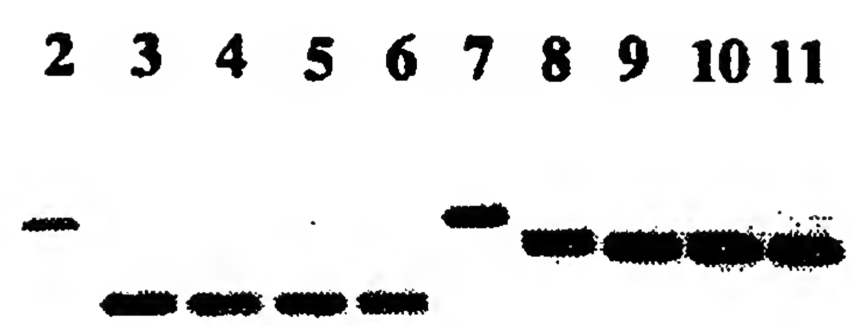
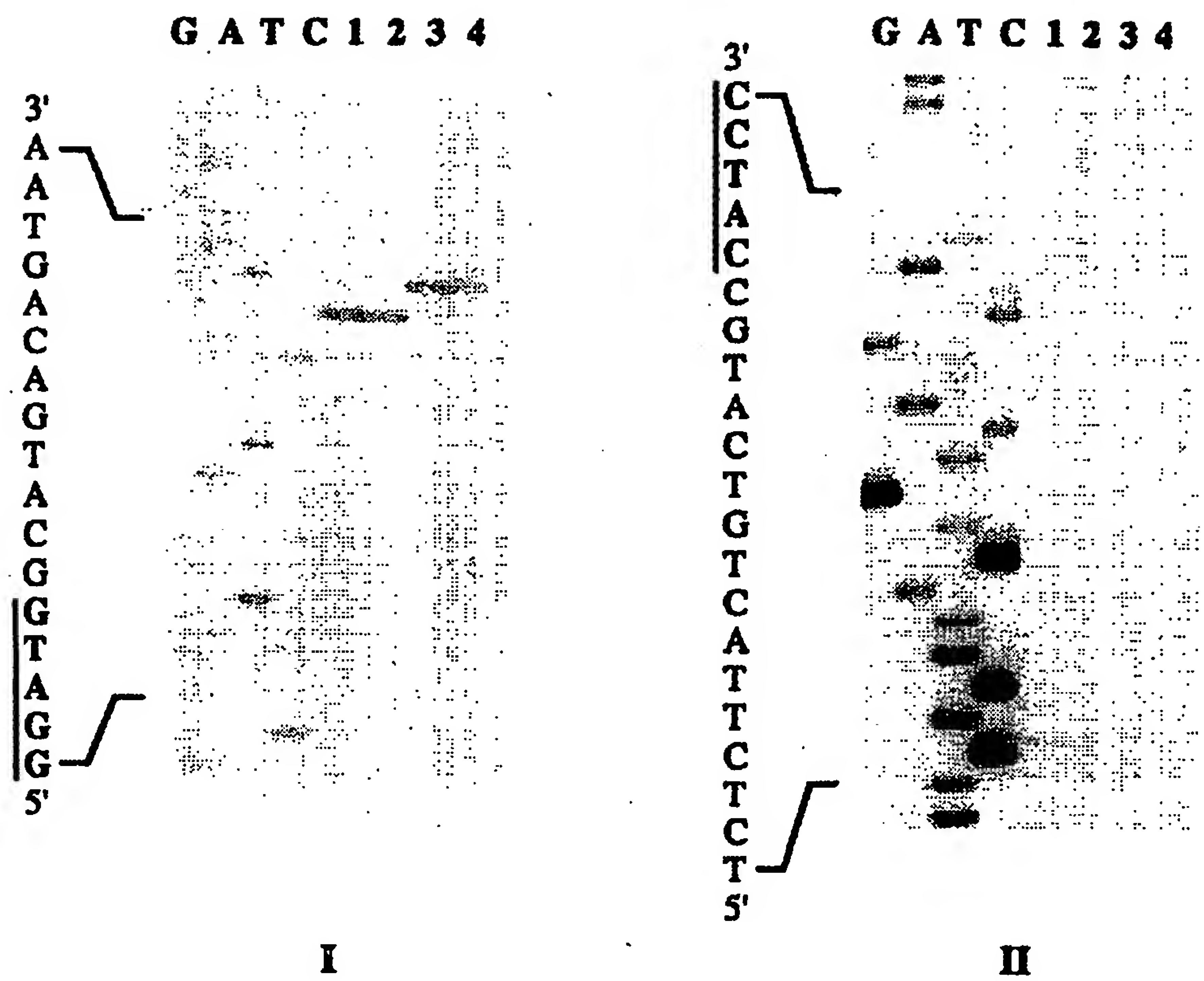


FIG. 14A


G A T C 1 2 3 4
3'
A
A
T
G
A
C
A
G
T
A
C
G
G
T
A
G
G
5'
I
G A T C 1 2 3 4
3'
C
C
T
A
C
C
G
T
A
C
T
G
T
C
A
T
T
C
T
C
T
5'
II

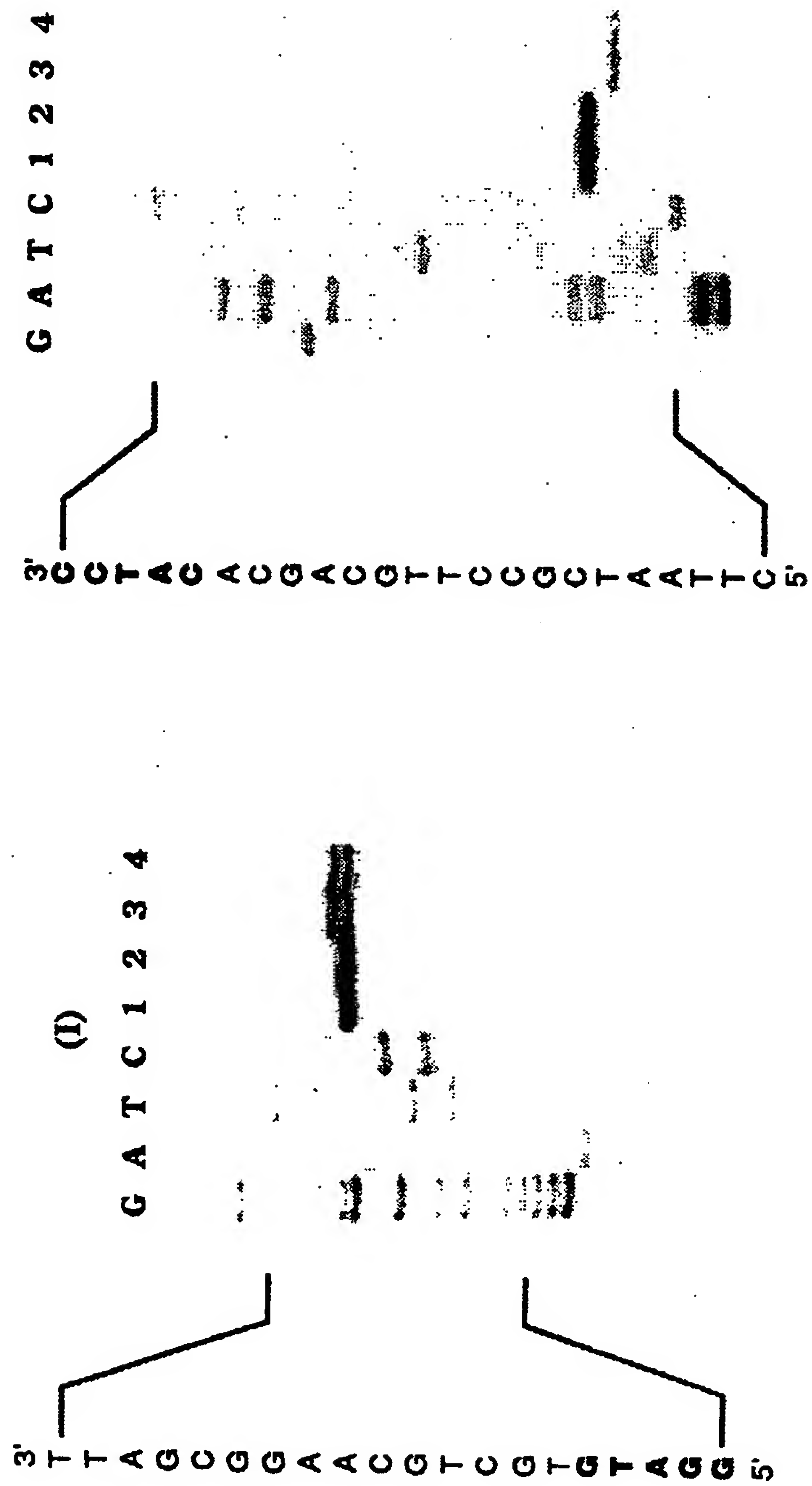

(I)
G A T C 1 2 3 4
3' T T A G C G G A A C G T C G T G T A G G 5'
(II)
G A T C 1 2 3 4
3' C C T A C A C G A C G T T C C G C T A A T T C 5'

FIG. 14B

FIG. 15A

(A) wild-type *Fok*I

5'- GGATGNNNNNNNNNNNNNNNNNN -3'
3'- CCTACNNNNNNNNNNNNNNNNNN -5'

FIG. 15B

(B) 4-codon insertion mutant

5'- GGATGNNNNNNNNNNNNNNNNNNN -3'
3'- CCTACNNNNNNNNNNNNNNNNNNN -5'

FIG. 15C

(C) 7-codon insertion mutant

5'- GGATGNNNNNNNNNNNNNNNNN -3'
3'- CCTACNNNNNNNNNNNNNNNNN -5'

FIG. 16

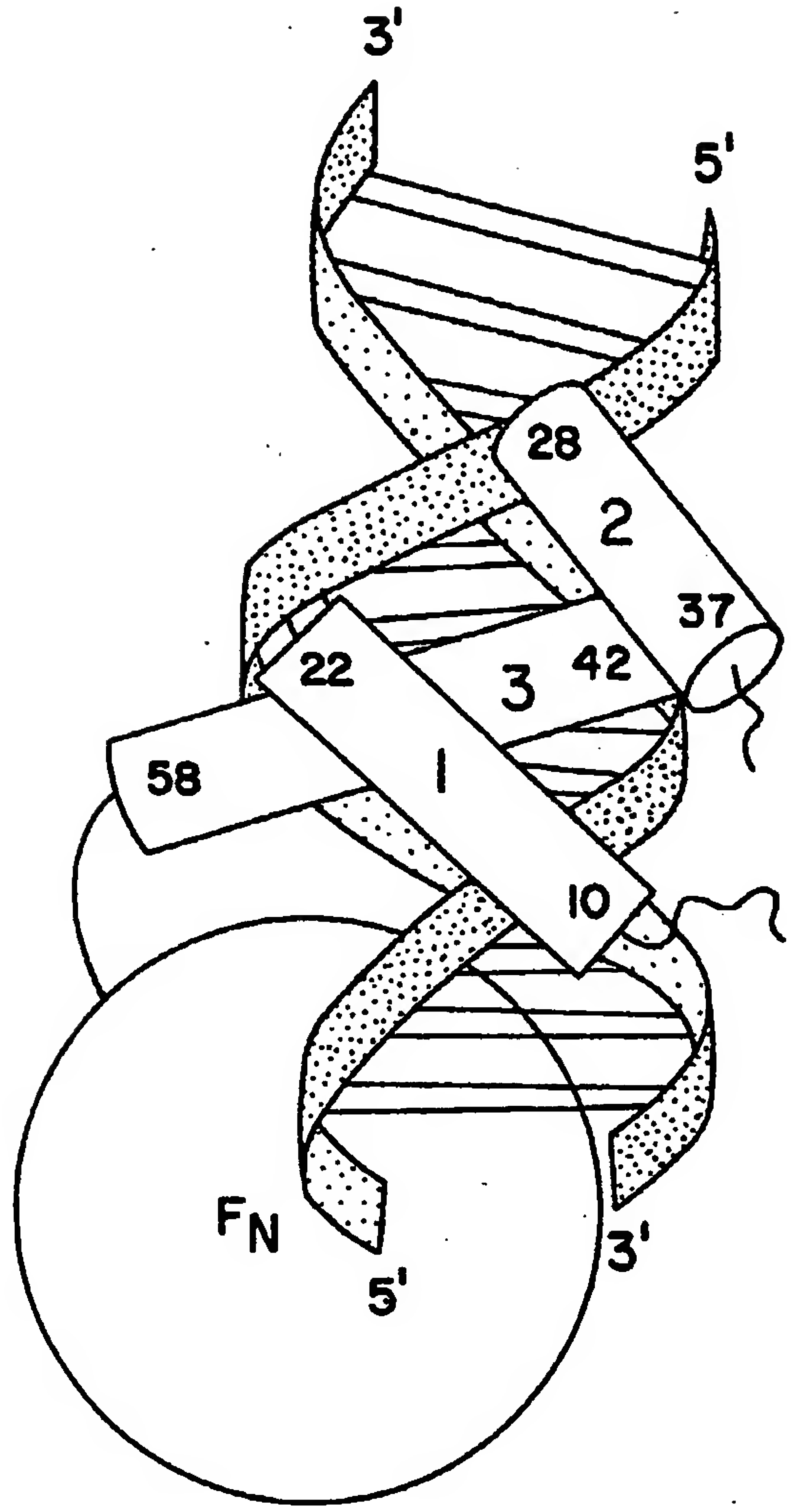


FIG. 17A

Ubx

```
                          PstI
5' - primer: 5' - TAC  CTGCAG C GGAGGT TTAAAAT ATG  CGA  AGA  CGC  GGC  CGA - 3'
                                               Met  Arg  Arg  Arg  Gly  Arg

                                                              SpeI
3' - primer: 3' - T  TAC  TTC  GAC  TTC  TTC  CTC  TAG  GTT  GAT  CAGAT - 5'
                     Met  Lys  Leu  Lys  Lys  Glu  Ile  Gln  Leu
```

Ubx-FN

```
                              NdeI
5' - primer: 5' - CCA  CGG  CAT  ATG  CGA  AGA  CGC  GGC  CGA - 3'
                                 Met  Arg  Arg  Arg  Gly  Arg

                                                                BamHI
3' - primer: 3' - TTA  TTG  CCG  CTC  TAT  TTG  AAA  ATT  ACT  CCTAGG  AT - 5'
                  Asn  Asn  Gly  Glu  Ile  Asn  Phe
```

FIG.17B

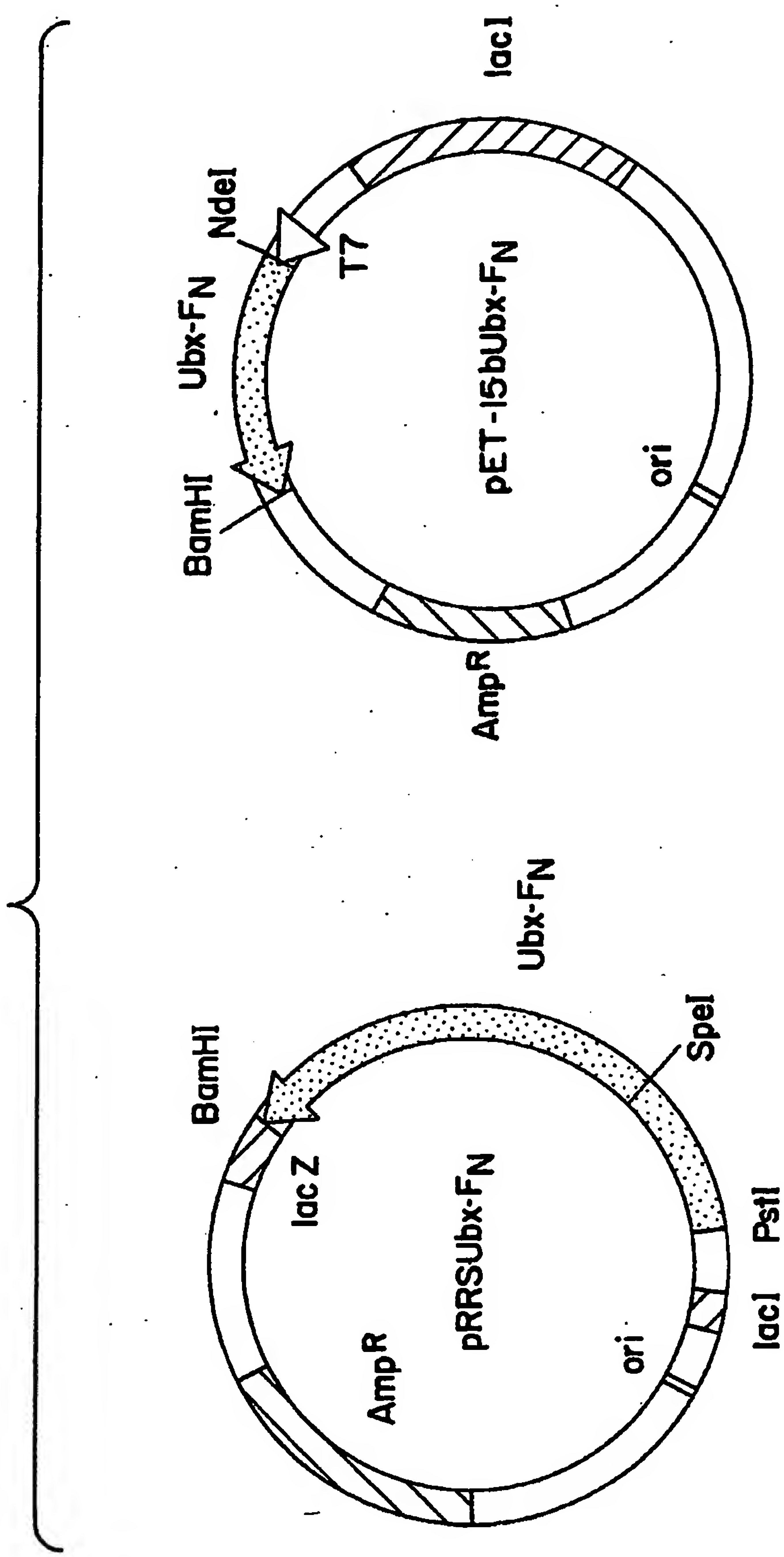

lacI
NdeI
T7
Ubx-F_N
pET-15bUbx-F_N
BamHI
ori
Amp^R
Ubx-F_N
BamHI
SpeI
lac Z
pRRSUbx-F_N
PstI
Amp^R
ori
lacI

FIG. 18

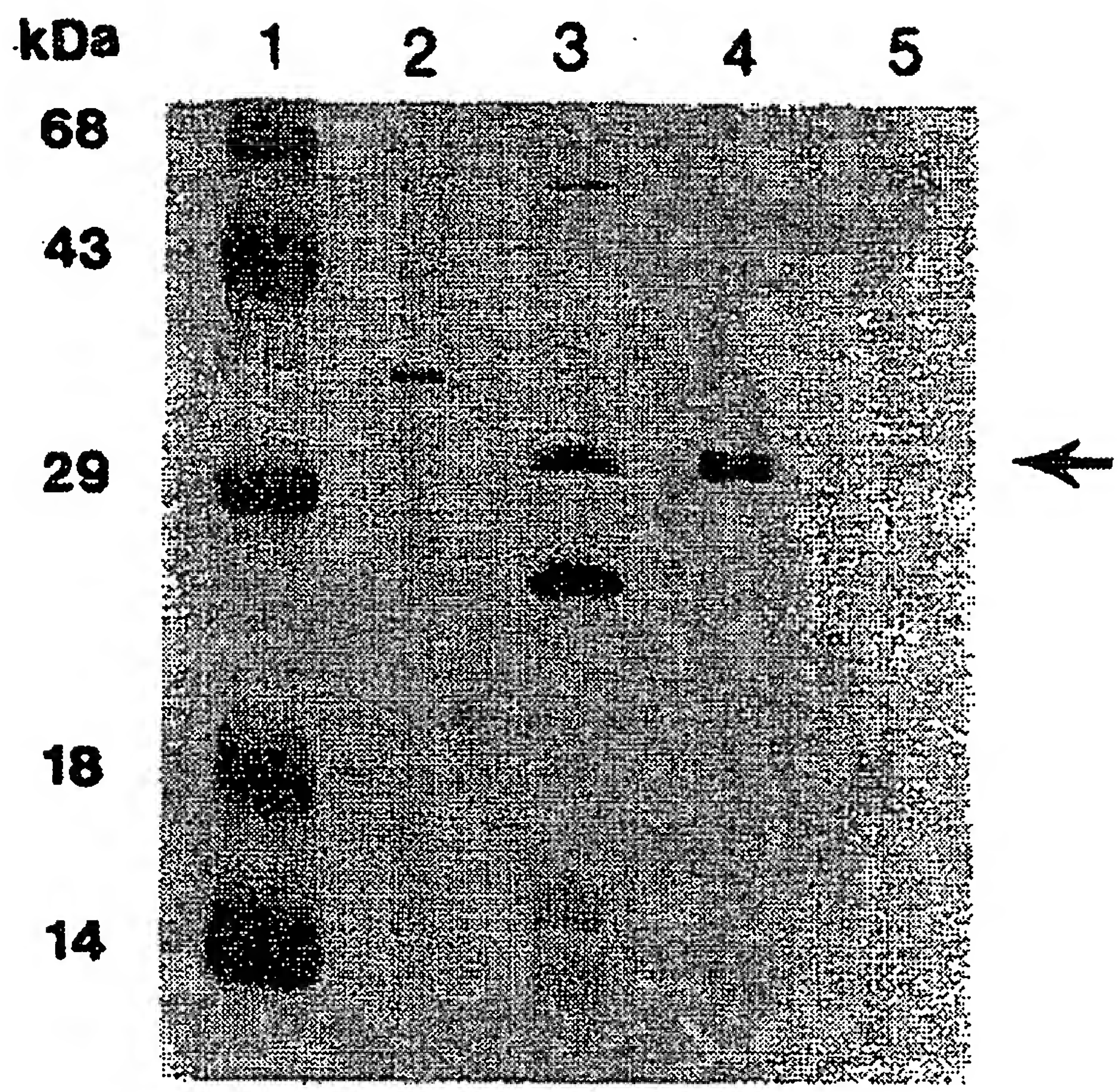


FIG. 19A

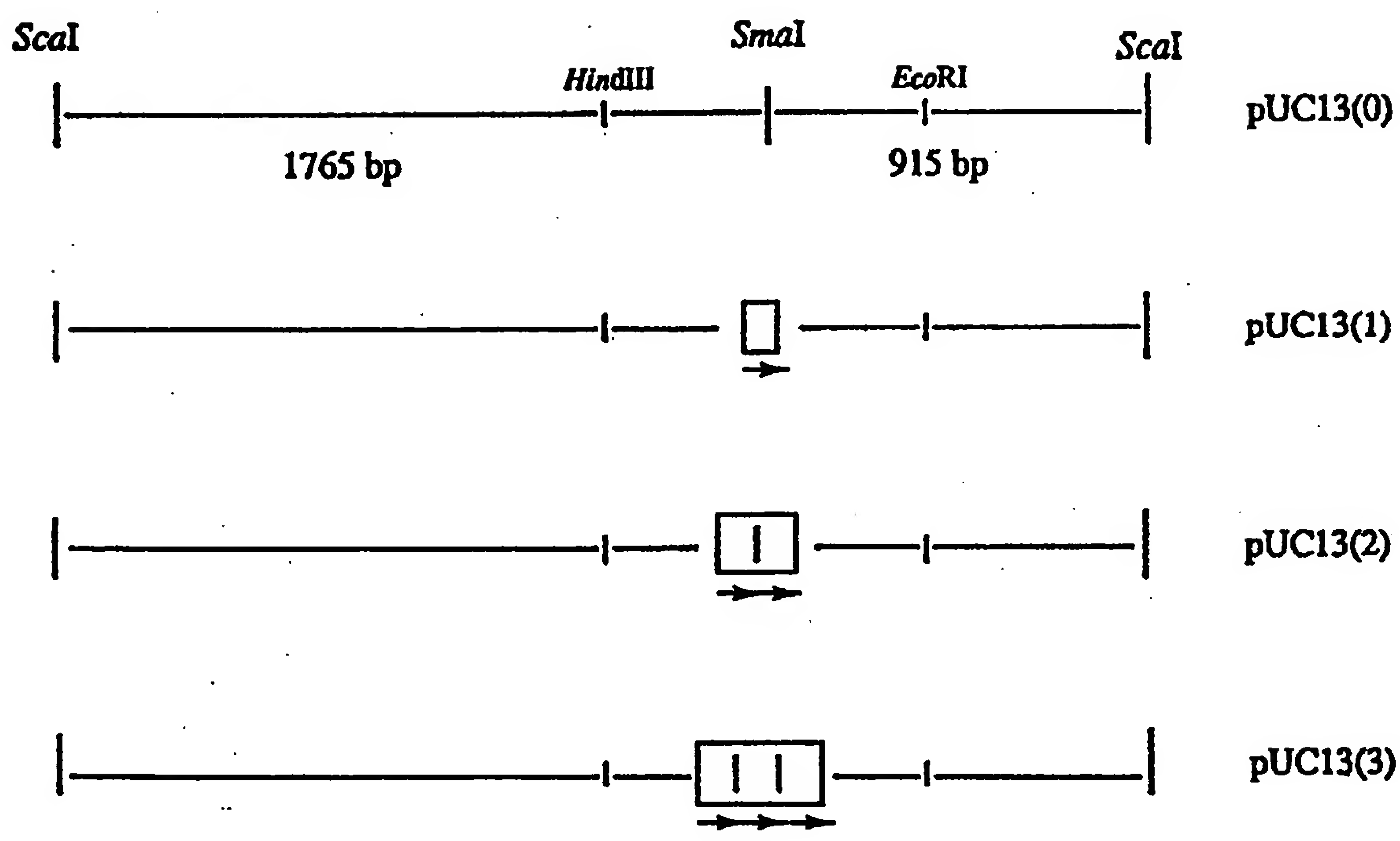

ScaI
HindIII
SmaI
EcoRI
ScaI
1765 bp
915 bp
pUC13(0)
pUC13(1)
pUC13(2)
pUC13(3)

FIG. 19B

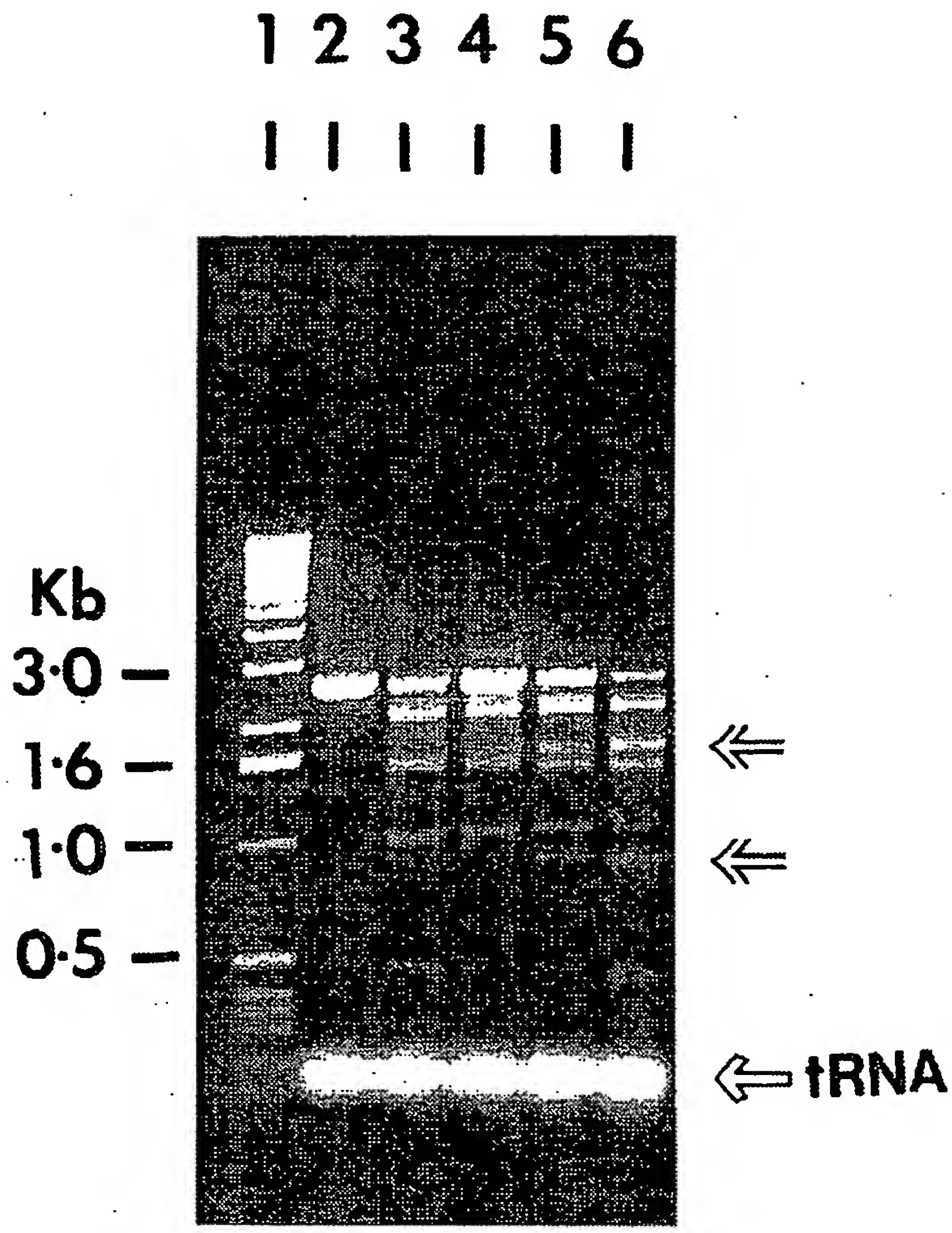


FIG. 20A

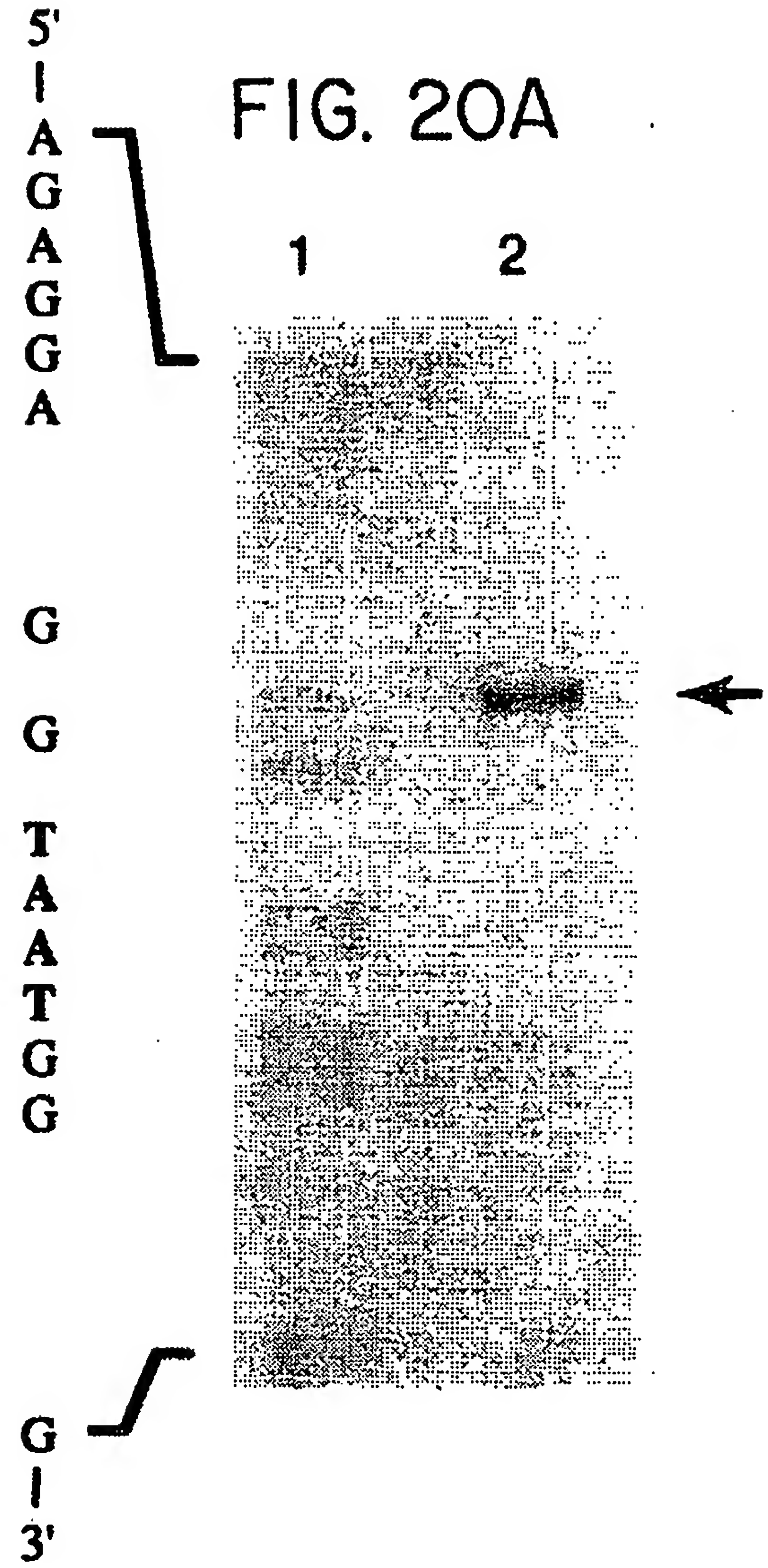
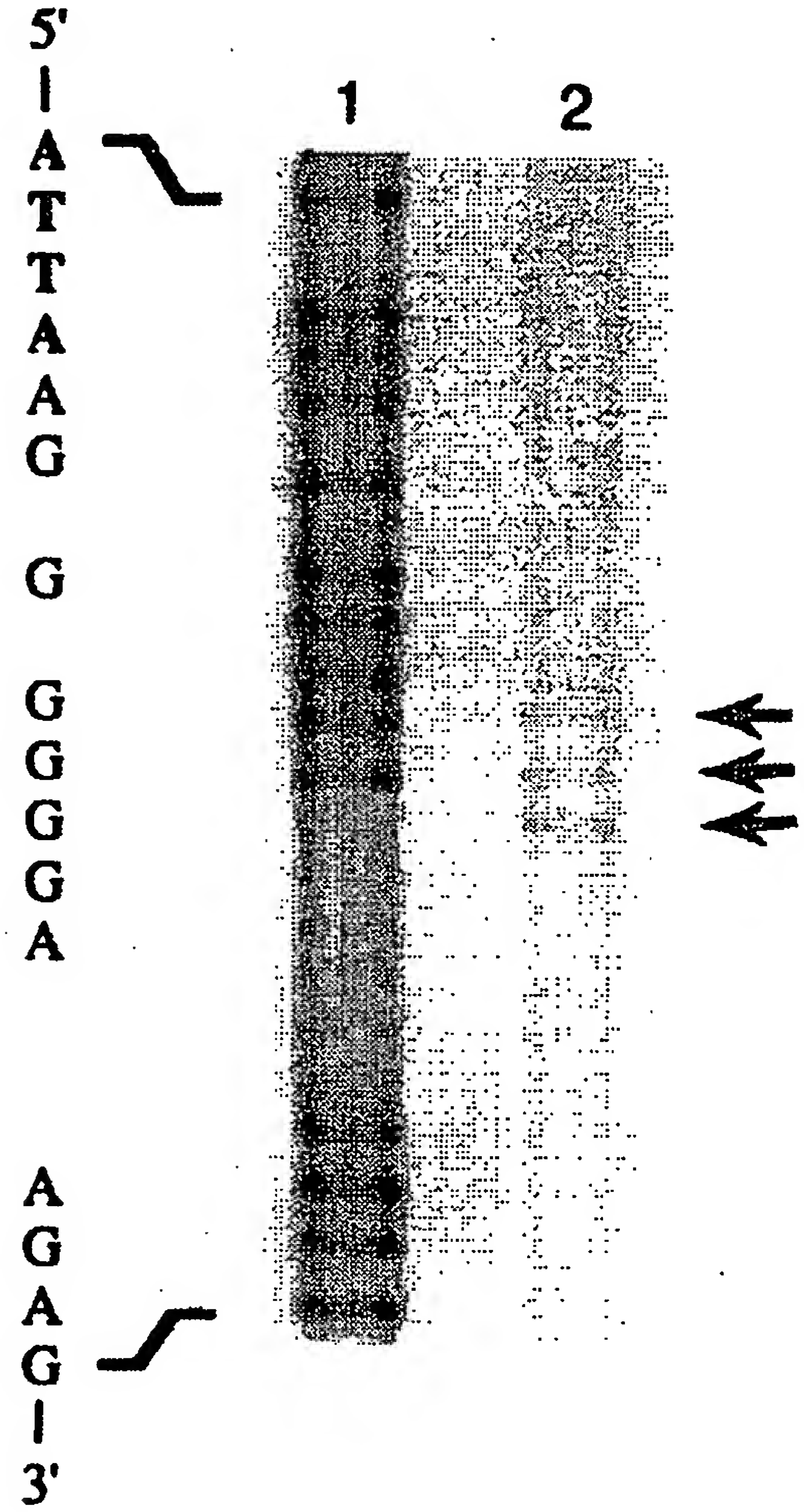


FIG. 20B



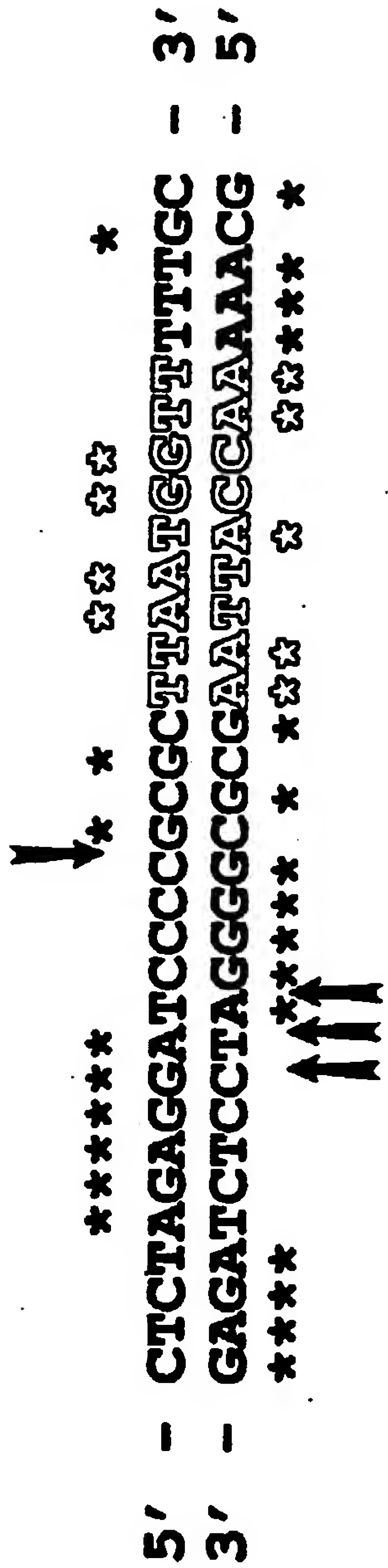

5′ - CTCTAGAGGATCCCCGCGCTTAATGGTTTTTGC - 3′
3′ - GAGATCTCCTAGGGGCGCGAATTACCAAAAACG - 5′

FIG. 20C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09143

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12N 9/22, 15/55, 15/70
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Computer Search - CA and APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	Proc. Natl. Acad. Sci. USA, Volume 89, issued May 1992, L. Li, et. al., "Functional Domains In *Fok*I Restriction Endonuclease", pages 4275-4279, especially page 4279, column 2.	1-12, 34-37 — 13-33
Y	Nucleic Acids Research, Volume 20, No. 16, issued 25 August 1992, K. Kita, et. al., "Cloning And Sequence Analysis Of The StsI Restriction-Modification Gene: Presence Of Homology To *Fok*I Restriction-Modification Enzymes", pages 4167-4172, especially page 4167, column 2.	1-37

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 SEPTEMBER 1994	12 DEC 1994

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Charles Patterson
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09143

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nucl. Acids Res., Volume 19, No. 5, issued 11 March 1991, H. Bocklage, et. al., "Cloning And Characterization Of The *Mbo*II Restriction-Modification System", pages 1007-1013, especially page 1007, column 2.	1-37
A	J. Biol. Chem., Volume 264, issued 5 April 1989, K. Kita, et. al., "The *Fok*I Restriction-Modification System. I. Organization and Nucleotide Sequences of the Restriction and Modification Genes", pages 5751-5756.	1-37
Y	Gene, Volume 80, issued 1989, M.C. Looney, et. al., "Nucleotide Sequence Of The *Fok*I Restriction-Modification System: Separate Strand-Specificity Domains In The Methyltransferase", pages 193-208.	1-37
Y	EMBO J., Volume 10, No. 5, issued 1991, S. C. Ekker, et. al., "Optimal DNA Sequence Recognition By The Ultrabithorax Homeodomain Of *Drosophila*", pages 1179-1186.	13-33
Y	EMBO J., Volume 11, No. 11, issued 1992, S. C. Ekker, et. al., "Differential DNA Sequence Recognition Is A Determinant Of Specificity In Homeotic Gene Action", pages 4059-4072.	13-33

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09143

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/199, 69.7, 252.33
536/23.2

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

435/199, 69.7, 252.33, 193
536/23.2
935/47